UNIVERSITÀ DEGLI STUDI DI GENOVA

CEBR

Center of Excellence for Biomedical Research

Dottorato in Immunologia Clinica e Sperimentale

Ciclo XXXIII

Dynamics of peripheral T populations after chemo- and/or radiotherapy in HNSCC patients

Tutor:

Chiar.ma Prof.ssa Daniela Fenoglio

Co-tutor:

Chiar.mo Prof. Dott. Gilberto Filaci

PhD student:

Sara Vlah

Abstract

Head and neck squamous cell carcinoma (HNSCC) is a relevant medical issue since it is the sixth most common cancer globally and its incidence is increasing. It is is generally associated with a history of alcohol and tobacco consumption or abuse, and remarkable an etiopathogenetic correlation between tumor and Human Papilloma Virus (HPV). Surgery, radiotherapy and chemotherapy in various combinations are used in the treatment of head and neck cancers, depending on the TNM stage. Chemo-radiotherapy mechanisms of action against tumors considers the activation of anti-cancer immune response as a key event, since radiations act as a sort of "in situ vaccination". Radiotherapy promotes the inflammation and subsequent activation of adoptive immune responses against tumor-associated antigens (TAA) released or expressed by the tumor. The peripheral immune response of 20 patients suffering for HNSCC was monitored using multiparametric flow cytometry in order to define the qualitative and quantitative characteristics of their immunological T cell response (CD4+ and CD8+ T lymphocytes maturation stage; cytokine production; regulatory commitment; expression of markers of exhaustion/IRs), before and after these subjects underwent chemo-radiotherapy treatment. The immunological monitoring data collected suggest an activatory effect on the immune system after therapy possibly due to the huge amount of cell death products acting as danger signals raised by chemotherapy-Rt combination and by the subsequent stimulation of cancer specific immune responses induced by antigen spreading. Peripheral blood samples of patients examined showed that therapy mainly induced a decrease of the CD3+ total T cell; the CD4+ T cells switched from the naïve to the late maturation stages cell subset expressing the PD-1 molecule, associated with an increase in secretion of Granzyme; on the CD8+ T cell compartment the therapy induced pro-maturation effects pushing them toward acquisition of full effector functions, suggested by the expansion of the CD8+CD28- T cell subset and by the decrease of the early maturation stages associated with the increase of Terminal Effector Memory CD8+ T.

Index

1. Introduction

1.1 Head and Neck Squamous Cell Carcinoma (HNSCC)

Head and neck squamous cell carcinoma (HNSCC) is a relevant medical issue since it is the sixth most common cancer globally and its incidence is increasing. Malignant tumors of the head and neck represent about 3% of all malignant tumors in Italy; approximately 12,000 new cases are diagnosed each year. The incidence rate is 16 cases per 100,000 Italians per year, while in Europe it is 18 per 100,000. Almost 90% of the malignant tumors of the head and neck are represented by squamous cell carcinomas which can have an extremely variable biological behavior in the different locations of the cervico-cephalic area [1].

Tumors of the oropharynx (OPSCC) are relatively frequent in the head and neck area. Based on existing cancer registries, the incidence in Italy in the period 1995-2002 was 2.58 cases per 100,000 inhabitants. Men are affected more than women (M: F = 6: 1) especially between the ages of 50 and 70. In recent decades, the incidence of tumors related to the infection of the Papilloma virus (Human Papilloma Virus HPV) has significantly increased. [2].

Head and neck squamous cell carcinoma (HNSCC) is generally associated with a history of alcohol and tobacco consumption or abuse, which are predisposing factors also for other tumors of the upper respiratory tract, thus favoring a not negligible incidence of multiple synchronous or metachronous tumors [3].

The etiopathogenetic correlation between HPV infection and the appearance of squamous cell carcinomas of the oropharynx (especially of the palatine and lingual tonsil) with a relatively favorable prognosis is now well established. Patients with HPV + tumors are generally younger

than those with HPV-negative, less comorbid, socially active tumors, so preserving quality of life after treatment becomes a priority [4].

Surgery, radiotherapy and chemotherapy in various combinations are used in the treatment of head and neck cancers, depending on the TNM stage.

The early stage ones (T1-2, N0-1) can be treated with surgery - more specifically trans-oral or open resection, with or without neck dissection or with radical radiotherapy.

Relating to locally advanced stage cancers (T3-4, N0-1 or any T, N2-3), radiotherapy associated with cisplatin-based chemotherapy is considered the standard, as chemo-radiotherapy compared to radiotherapy alone has shown a significant improvement in overall survival, locoregional control and disease free survival [5-6].

1.2 Immune response to cancer

Cancer result from uncontrolled proliferation and spread in the body of clones of transformed cells, characterized by the accumulation of a variable number of genetic alterations and the loss of normal cellular regulatory processes.

The immune system takes advantages of at least three functions to try to prevent the development of tumors: a) protection of the host from viral infections and therefore from virus-induced tumors; b) prevention of the establishment of an inflammatory microenvironment favoring carcinogenesis by eliminating pathogens and therefore rapidly resolving inflammatory state; c) elimination of transformed cancer cells by specific immune cells. [7-8-9]

Recent studies showed how the immune system recognizes tumor cells through tumor-associated antigens (TAA), a large category that includes: differentiation antigens (such as melanocyte differentiation antigens), mutational antigens (such as p53), overexpressed cellular

antigens (such as HER-2), viral antigens (such as human papillomavirus proteins), and cancer/testis (CT) antigens that are expressed in germ cells of testis and ovary but silent in normal somatic cells (such as MAGE and NYESO-1).

In recent years it has become evident that the immune system plays a dual role in relation to cancer: on one side, immune system has the ability to protect the host against tumor formation (immunosurveillance), on the other side can enhance tumor-promoting actions and modulates the immunogenicity of cancer cells (editing). This complex relationship between immune system and tumor give rise over time to a consolidated hypothesis that takes the name of "cancer immunoediting".

The cancer immunoediting process can be distinguished in three phases: 1) Elimination, 2) Equilibrium, 3) Escape. (FIG.1)

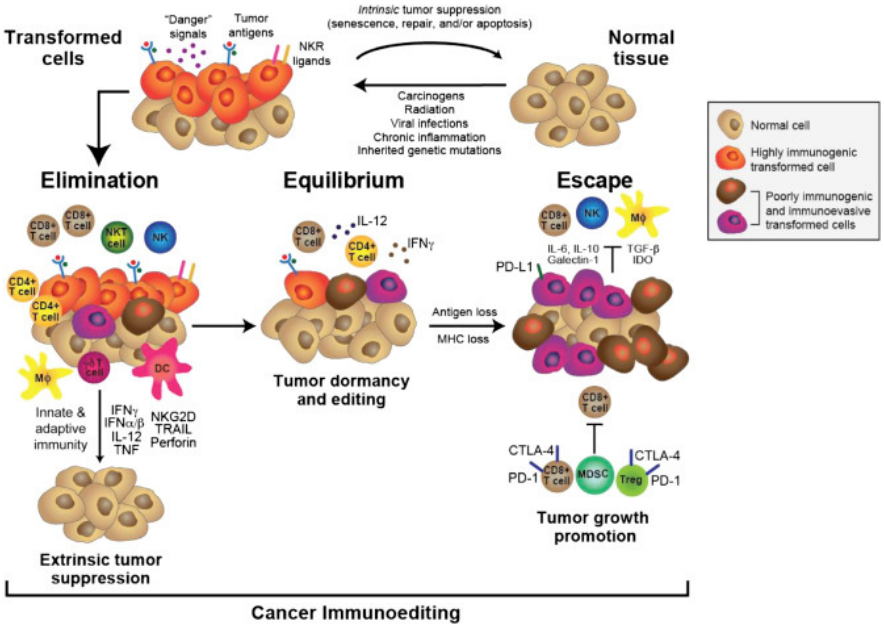

FIG.1. Cancer immunoediting process A dynamic relationship is established between the immune cells and the tumor, called immunoediting, which reflects the dual role of the immune system in promoting the protection of the host from the tumor and in favoring tumor escape versus immune destruction. The immunoediting process is divided into three phases: 1) elimination of tumor cells thanks to immunosurveillance; 2) phase of equilibrium in which the tumor cells are controlled but not eliminated; 3) escape phase in which the modification of cancer cells involves loss of the immune response and favors the progression of the disease. doi: 10.1146/annurev.immunol.22.012703.104803.

During the elimination phase, the innate and adaptive immune systems work together to detect the presence of a developing tumor and destroy it before it becomes clinically apparent. Many molecules, that act as "Danger signals", are induced early during tumor development: Type I IFNs activate dendritic cells and promote induction of adaptive anti-tumor immune responses; damage-associated molecular pattern molecules (DAMPs) released directly from dying tumor cells [such as high mobility group box 1 (HMGB1)] or from damaged tissues (such as hyaluronan fragments), as well as stress ligands, that are frequently expressed on the surface of tumor cells, activate receptors on innate immune cells, which release pro-inflammatory and immunomodulatory cytokines, which in turn establish a microenvironment that facilitates the development of a tumor-specific adaptive immune response that requires the additional expression of tumor antigens capable to promote the expansion of effector CD4+ and CD8+ T cells.

In the equilibrium phase, the host's immune system and the tumor cells, that survive the elimination phase, enter into a dynamic equilibrium, in which the lymphocytes and IFN-γ exert a powerful selection pressure on the tumor cells that is sufficient to contain, but not completely extinguish the tumor containing many genetically unstable and mutant tumor cells. This is the longest phase of the three processes of cancer immunoediting and can last for many years. The end of equilibrium process hesitates into a new population of tumor clones with reduced immunogenicity, derived from a heterogeneous parental population by the modeling forces of the immune system.

Finally, in the escape phase, tumor cells acquire the ability to circumvent immune recognition and destruction. Progression from equilibrium to the escape phase can occur because the tumor cell population changes in response to the immune system's editing functions and/or because the host immune system changes in response to increased cancer-induced immunosuppression or immune system deterioration. Tumor cell escape can occur through many different

mechanisms: a)- loss of tumor antigen expression, which involves MHC class I molecules; b) production of immunosuppressive cytokines (as IL-10, TGF-β); c)-recruitment of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs). [10-11-12]

The importance of T lymphocytes in all their forms in the cancer immunoediting process, especially as infiltrating CD8 + T cells, has been demonstrated in many preclinical mouse models and in human tumors. In recent years, the study of the T populations of both the peripheral district and the tumor site by the different technological platforms has assumed particular significance to monitor the dynamic of T cells for the prognosis and prediction of therapy responses.

1.3 T lymphocytes

T lymphocytes derive, just like B lymphocytes, from the multipotent haematopoietic progenitor which migrates from the bone marrow to the thymus (hence the name thymus-dependent lymphocytes) where it undergoes a complex maturation process. Through a sophisticated mechanism of elimination of T cells potentially autoreactive or unable to recognize the antigens presented by MHC-molecules in the thymic stroma which implies the positive and negative selections, the final result consists of the generation of a mature conventional CD4+ and CD8+ T cell repertoire that is both MHC-restricted and tolerant towards autologous peptides. The hallmark of a T cell is expression of an antigen-recognizing T cell receptor (TCR), composed of: an α - and β -chain (TCR α: β) expressed by 95% of peripheral CD3+ T cells or and a γ- and δ – chain (TCR γ: δ) expressed by 5% of peripheral CD3+ T cells. [13-14]

1.4 T effector cells and Maturation

The mature CD3+T cell population appears extremely heterogeneous and can be divided into distinct subsets based on their phenotype. The association between distinct subsets and the expression of surface receptors and intracellular molecules implies that these T cell subsets exhibit differential requirements for stimulation and survival, homing potential (e.g., to lymphoid organs or to peripheral tissues) and some (immediate) effector functions.

Human naïve and memory T cells can be identified by the reciprocal expression of the most-commonly used markers: CD45RA (or the isophorm CD45RO) and CCR7, a chemokine receptor, which allows them to be recycled in the areas reserved for T lymphocytes within peripheral lymphoid tissues.

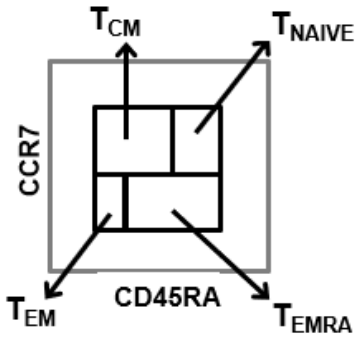

FIG.2. Diagram of the maturation stages of T lymphocytes based on the expression of CD45RA and CCR7.

Staining of peripheral blood T cells with antibodies to CD45RA and CCR7 revealed four subsets of CD4+ cells ad well as CD8+ cells (Fig.2):

- T Naïve (CD45RA+CCR7+), that have not previously contacted their relevant antigen. Naïve T cells are programmed to recirculate from blood into peripheral lymphoid tissues and then back to blood;

- Central memory T cells or T_{CM} (CD45RA-CCR7+) are very sensitive to the activation of their receptors, rapidly expressing the CD40 ligand in response; however, they are slower than other subsets in acquiring effector functions, such as the production of cytokines;
- T Effector Memory or T_{EM} (CD45RA- CCR7-) even if they lack the CCR7 chemokine receptor, this cells subset express high levels of β1 and β2 integrins, are therefore able to rapidly enter inflamed tissues, and have the ability to rapidly assume effector function and thus secrete large amounts of IFN-γ, IL-4 and IL-5 upon stimulation;
- Terminal Effector Memory or T_{EMRA} (CD45RA+ CCR7-) which consists of those effector cells which are terminally differentiated and which, however, return to express the CD45RA a marker that is typically found on T Naïve. [15-16- 17]

Regardless of each maturation stage, the recognition of the antigen and the consequent response to it, is subjected to a series of primary and secondary signals, so that is a tightly controlled process at every step of a mature T cell response.

Primary signal for conventional T cells is mediated through TCR engagement. Conventional T cells through their TCR recognize small antigenic peptides presented in the groove of the self major histocompatibility complex (MHC) by antigen presenting cells (APC). As a result of this recognition, TCR complexes aggregate on T cell surfaces to form stable contacts resulting in the formation of immunological synapses on APC. Early intracellular signaling, following TCR engagement, involves the activation of protein tyrosine kinases (PTK), leading to the phosphorylation of CD3-localized immunoreceptor tyrosine-based activation motifs (ITAMs). Subsequently, the PTK ZAP-70 is recruited, resulting in a series of phosphorylation events.

After recognition of peptide–MHC complexes on APCs, secondary signal receptors colocalize with TCR molecules at the immunological synapse, where they promote or inhibit T cell activation and function. In fact, the balance between co-stimulatory receptors and inhibitory

receptors (iRs) allows to control the T cell response at every step, from T cell priming, T cell expansion, and contraction, to the various functions of memory T cells. [18]

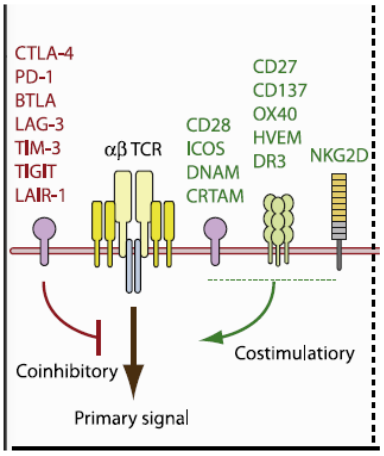

Fig.3.Cell Surface Signaling Molecules in the Control of Immune Responses [18]

Expression of co-stimulatory and inhibitory molecules on the surface of T cells (Fig.3) is induced following activation and that changes in the cell surface expression of many molecules occur in overlapping fashion as T cells proliferate and differentiate. [19]

Co-stimulatory receptors, such as CD28 and CD27 molecules are involved respectively in the regulation of T-cell activation and in the generation of antigen-primed cells. Both CD28 and CD27 markers are constitutively expressed on the cell surface of naive CD4+ and CD8+ T cells and in particular CD28 provides an essential co-stimulatory signal for T cell growth and survival upon ligation by B7-1 and B7-2 molecules on APCs. The expression of CD28 and CD27 markers are modulated in the later stages of maturation and activation (T_{EM} and T_{EMRA}); CD28 has a role in memory T cell survival, and with CD27 promotes memory CD4+ and CD8+ T cell responses. During the peak of T cell activation, the expression of co-stimulatory receptors is replaced by the expression of co-inhibitory receptors that function to suppress T cell activities.

Co-inhibitory or inhibitory receptors (iRs) are molecules that negatively interfere with T cell activation. Their primary role regards the regulation of immune homeostasis and preservation of healthy tissue from autoimmunity. They function at several levels: (1) through competition with co-stimulatory receptors for binding to shared ligands or interference in the formation of microclusters and lipid rafts; (2) by interfering with downstream signals from co-activatory and T cell receptors (TCRs); (3) by upregulating genes that are involved in T cell dysfunction (Fig.4). [18-20-21]

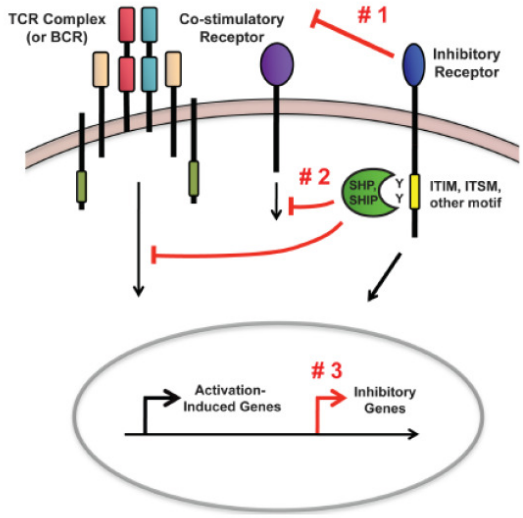

Fig.4 Major inhibitory receptor mechanisms of action. Mechanism#1: Inhibitory receptors prevent T cells (or B cells) from receiving complete activation signals by sequestering the ligands for costimulatory receptors. Mechanism #2: Inhibitory sequence motifs, such as ITIMs or ITSMs, on the cytoplasmic tail of inhibitory receptors are phosphorylated upon cellular activation. #3: Inhibitory receptors were recently demonstrated to upregulate genes that inhibit immune cell function; however, the pathways leading to this gene upregulation are not known. **[22]**

Two of the probably most well-studied T cells related iRs are Programed Cell Death 1 (PDCD1, also called PD-1) and Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4). CTLA-4 and PD-1 are crucial for the maintenance of central and peripheral tolerance.

CTLA-4 is up-regulated following T cell activation and is involved in later stages of T cell priming and systemic activation of T cell responses. CTLA-4 counteracts the positive signal mediated by CD28 by competing for the same ligands (CD80/86) with higher affinity. CTLA-4 is up-regulated upon activation on naïve T cells and constitutively expressed on regulatory T cells. Paradoxically, although CTLA-4 decreases effector functions of CD4+ and CD8+ T cells, it increases the suppressive capacity of Tregs. Thus, CTLA-4 dampens T cell activation, decreases the efficacy of antigen presenting cells to activate T cells and augments Treg mediated immune suppression. Whereas CTLA-4 predominantly regulates initial T cell activation, the inhibitory receptor programmed cell death 1 (PD-1) dampens effector T cell functions, which are which are typically carried out by T cells in an advanced state of maturation. In fact, Transient PD-1 cell surface expression is initiated upon T cell activation and plays a role at later stages of the immune responses when T cells are already activated. [19]

Beside iRs activity, other pathways work in order to prevent inappropriate activation of the immune system. One of these is the co-inhibitory pathways associated with purinergic signaling with the focus on CD39 and CD73, extracellular nucleotide scavenging, and adenosine generation.

Extracellular release of purine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), plays a fundamental role in regulating inflammation and tissue homeostasis through the activation of purinergic P2 receptors. The conversion of ATP/ADP to adenosine is the consequence of the action of cell-surface ectonucleotidases CD39 and CD73.

CD39, or ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1), hydrolyses extracellular ATP and ADP into adenosine monophosphate (AMP), which is then processed into the anti-inflammatory adenosine, essentially by the ecto-50-nucleotidase CD73. Upon binding to A2A receptors on T cells, adenosine induces the accumulation of intracellular cyclic AMP,

thereby preventing TCR-induced CD25 upregulation and inhibiting effector T-lymphocyte proliferation and inflammatory cytokine secretion. [23-24-25]

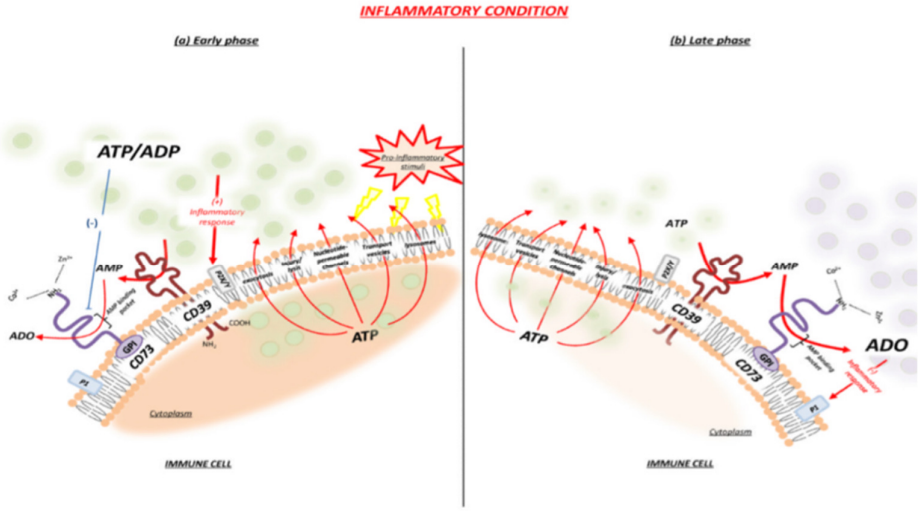

Fig.5 CD39 and CD73 shape the "purinergic halo" surrounding immune cells. The occurrence of pathological events, such as inflammation, promotes a massive accumulation of ATP, which serves as a key "danger" signal, triggering a series of proinflammatory responses [25]

1.5 T cells exhaustion

T cell exhaustion occurs as the result of persistent antigen stimulation and consequent inflammation state induced during chronic infections or cancer.

Exhaustion is intimately linked to concurrent analyses of functional effector and memory T cells and represent a distinct and dysfunctional state of CD8+ and CD4+ T cell differentiation. Exhausted T cells are thought to develop during the progression from effector T cells to memory T cells, so it has been proposed that exhaustion of T cells may prevent the terminal differentiation of effector T cells to memory cells. (Fig.6)

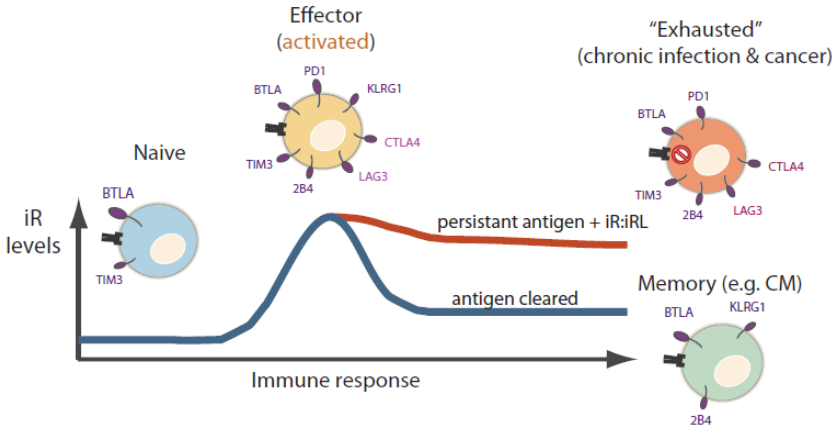

Fig.6 Levels of iR may peak at the effector phase, and may further modulate differently during acute versus chronic immune responses. [20]

During exhaustion, T cells progress through stages of dysfunction in a hierarchical manner, losing effector functions and other properties, becoming gradually silenced due to continuous T cell receptor (TCR) stimulation, resulting in impaired effector cytokine production (e.g., IFNg, TNFa, or IL-2), and impaired ex vivo cytotoxicity compared with effector T cells. [26-**27**-28]

Hallmarks of exhaustion mechanisms are: a) the elevated expression of multiple co-inhibitory receptors (PD-1, TIM-3, CTLA-4, BTLA, CD160, LAG-3) which in turn highly correlates with the

degree of unresponsiveness, in particular, the axis of PD-1 and its ligand seems to be a major inhibitory receptor pathway involved; b) the role of immunoregulatory cytokines (such as IL-10 and TGF-β), and immunoregulatory cell types (such as Treg cells and other cells). Hence, immunoregulation is centrally involved in T cell exhaustion. [26]

1.6 T regulatory cells

Regulatory T (Treg) cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. However, they also limit beneficial responses for example limiting anti-tumour immunity. [29]

The mechanisms by which Treg lymphocytes regulate the immune response can be summarized in four fundamental "modes of action" (Fig.7):

1. suppression by inhibitory cytokines (IL-10, TGFβ, IL-35);

2. suppression by cytolysis;

3. suppression by metabolic interference as: deprivation of IL-2 which binds to high affinity IL-2 receptor (CD25) on Treg cells resulting in the apoptosis of effector T lymphocytes; and generation of immunosuppressive adenosine through degradation of ATP by the CD39 and CD73 ectonucleotidases; [30-31]

4. suppression by modulation of function or maturation of antigen presenting cells, through the IRs: LAG3 (which suppresses the maturation of dendritic cells) and CTLA-4 (which induces production of IDO by the dendritic cells) [29]

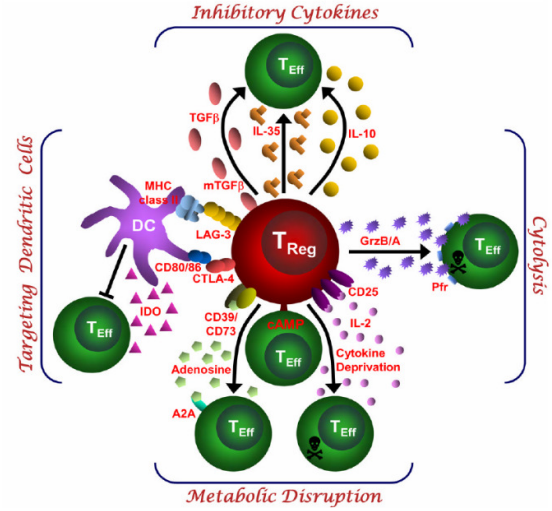

Fig.7 Operative modalities of the immunomodulatory function of Tregs: production of IL-10, TGFβ and Il-35 (top), direct elimination of T eff (right), metabolic destruction of T eff by means of CD25 (by deprivation of IL-2), CD39 and CD73 (below).

The action of Treg lymphocytes is important in preventing excessive inflammatory reactions when the antigen has been eradicated, thus preventing the activated lymphocytes from leading to tissue damage. On the other hand, in the context of neoplastic pathologies, an imbalance of the immune response in a suppressive sense would seem to give the tumor an advantage and therefore the possibility of prevailing (escape) over the cytotoxic immune response [32-33]

Several subsets of T cells with regulatory activities have been described.

Among CD4+ T lymphocyte subpopulations with immunomodulatory activity, it is possible to distinguish CD4+ $CD25^{high}$ Treg cells, so the cardinal feature of Treg cells subset is the constitutively expression high-affinity IL-2 receptor (CD25). [34].

Treg cells can be distinguished from recently activated T lymphocytes, that represents the 30% human peripheral blood and express low levels of CD25, by the expression of the nuclear transcription factor Forkhead box P3 (Foxp3) and the low intensity expression of the IL-7 receptor (CD127). [29].

Several studies have shown remarkable plasticity and heterogeneity of CD4+Treg populations and according on their site of differentiation CD4+Tregs are classified in: thymus-derived natural Tregs (nTregs), Foxp3+, that express marker of activation (HLA-DR) and functional markers (GITR, CTLA-4, CD39); and peripherally induced Tregs (pTregs), also FOXP3+, resulting from the conversion of naïve CD4+T cells in presence of interleukin-10 (IL-10) and/or transforming growth factor-β (TGF-β), as Th3 (first identified for their role in oral tolerance through the secretion of TGF-β) and Tr1, characterized on the basis of their role in preventing autoimmune colitis and their ability to secrete large amounts of IL-10. pTregs, generated in chronically inflamed tissues, are implicated in the induction of oral and gut tolerance. Moreover the In vitro generation of Tregs in the presence of IL-2 and TGF-β polarizing conditions leads to the development of iTregs FOXP3+ (Fig.8) [36]

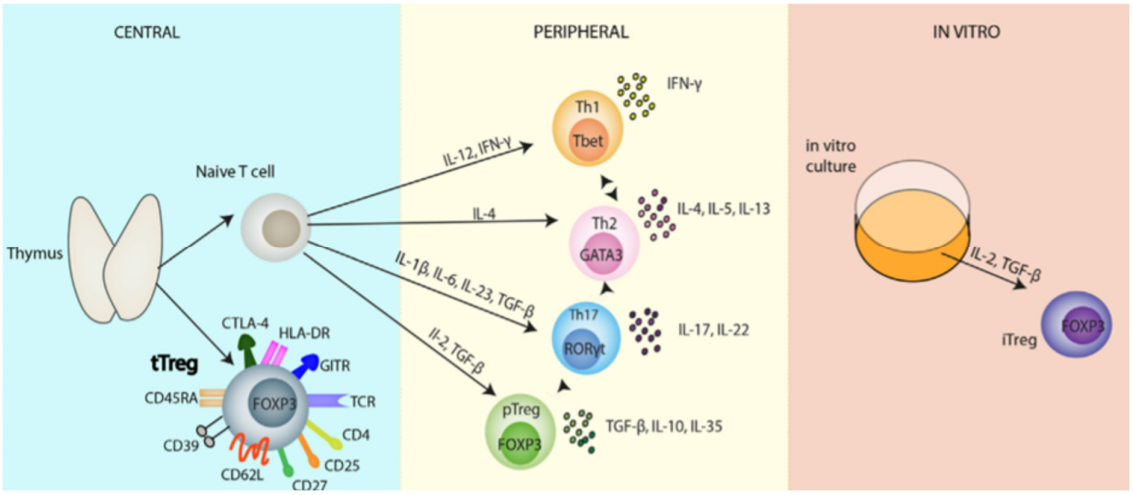

Fig.8 Regulatory T cell populations. The image shows the Treg cell populations. Natural Tregs are produced in the thymus together with naïve T which, subsequently, can differentiate in the periphery into different subsets of T cells: Th1, Th2, Th17, induced Treg, all having distinct immunological roles. The differentiation process is controlled by several cytokines. Th1 cells are induced by IL-2 and IFNγ; the Th2s differ thanks to IL-4; the Th17s thanks to IL-1β, IL-6, IL-23 and TGF-β. Each separate subset of T cells can be identified by specific transcription factors: T-bet (Th1), GATA3 (Th2), RORγt (Th17), FOXP3 (Treg) and has its own immunological role: Th1 secrete IFNγ, controlling the immunity to foreign pathogens. Th2s produce various cytokines including: IL-4, IL-5, IL-13, IL-10, which are mainly involved in promoting humoral immunity, protecting against infections. Th17s mainly produce IL-17 and IL-22. Despite the apparent terminal differentiation of all these cells, they absolutely cannot be considered definitive stages of cellular life. The possibility of changing phenotype during the exhaustion process is still the subject of research. In the presence of polarization conditions with IL-2 and TGF-β it is possible to generate iTreg. [36]

Furthermore, recent papers described a dissection of CD4+ $CD25^{high}$ FOXP3+ $CD127^{dim/neg}$ Treg cells in humans into three subpopulations based on expression levels of FOXP3 (or CD25) and the cell surface molecule CD45RA (Fig.9): (i) $FOXP3^{low}CD45RA+CD25^{low}$ cells, designated as naive or resting Treg cells; (ii) $FOXP3^{high}CD45RA–CD25^{high}$ cells, designated as effector or activated Treg cells, which are terminally differentiated upon antigen stimulation and exert their highly suppressive action expressing IRs including CTLA-4 and PD-1; and (iii) $FOXP3^{low}CD45RA–CD25^{low}$ non-Treg cells, which do not possess suppressive activity but can secrete pro-inflammatory cytokines. [32]

This classification delineates developmental stages of Tregs and assessing their adaptive processes in physiological and pathological immune responses.

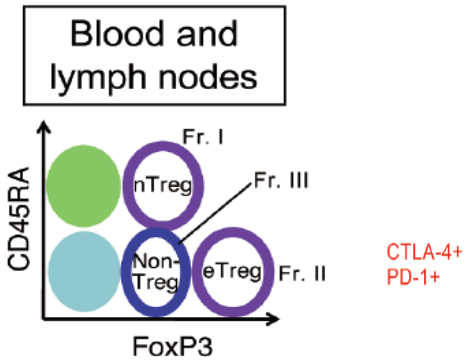

Fig.9 Functional classification of human FOXP3+CD4+ T-cell subpopulations. Human FOXP3+ T cells are composed of heterogeneous subpopulations containing suppressive Treg cells (naïve and effector Treg cells) and activated non-Treg cells without suppression function. These subpopulations are designated as Fraction (Fr.) I, II, and III for naive Treg (nTreg), effector Treg (eTreg), and non-Treg cells, respectively. CD25 surface marker can be used in the place of FOXP3 because of their correlative expression in humans. [32]

The regulatory T cell family also includes subsets belonging to the CD8+ T cell compartment. It is interesting to note that, both in humans and in the animal model, different CD8+ Treg subtypes have been identified that differ according to the molecular phenotype and the mechanism of action. Similarly to CD4+Tregs, thymus-derived or natural CD8+Treg populations have been described that are activated by direct antigen recognition and express the transcription factor FoxP3 and CD25: moreover they act generally through cell-to-cell contact.

Several types of peripherally induced CD8+Tregs have been described in humans induced after one time or several rounds of stimulation after loss of CD28 expression. The evidences suggest that the adaptive CD8+Tregs can originate from cells initially not regulatory and acquiring suppressor activity after repeated antigen stimulation in presence of polarizing milieu. [36-37] In particular, CD8+CD28- non-antigen-specific Treg cells appear to play a relevant role in tumor microenvironment, as well as in the pathogenesis of different autoimmune and infectious diseases [38]. These CD8+ Treg lymphocytes show a very low frequency (≤0.1% of peripheral CD3+T lymphocytes) in healthy donors, but they can be generated from circulating CD8+ lymphocytes following in vitro conditioning with IL-2 and IL-10. Whereas, in oncologic patient, active immunosuppressive CD8+Treg cells represent a large component of tumor- infiltrating T cells and their frequency in peripheral blood resulted significantly higher than that measured in the peripheral blood of healthy subjects .

The loss of co-stimulatory CD28 receptor suggests that they fall within the so-called "peripherally induced" CD8+ Treg [39]. The so far detailed phenotype of the regulatory subpopulation is CD8+CD28-CD127lowCD39+ and takes into account maturation state and functionality of this subset. Similarly to CD4+ $CD25^{high}$ FOXP3+ $CD127^{dim/neg}$ Treg cells, the maturation of circulating CD8+CD28- cells in regulatory cells is accompanied by the down-modulation of CD127. Several evidences showed that: 1) they are able to inhibit both cell

proliferation and cytotoxicity of effector T cells through the secretion of cytokines, mainly IL-10; 2) they do not require direct contact with the target cell to exert suppressive activity [40]; and 3) they act through regulatory mechanism that includes the activity of CD39. [31,41].

In conclusion, several T cell populations endowed with potent immunomodulatory capacities have been identified in both the CD4+ and CD8+ compartments. The fundamental difference between CD4 +and CD8+ T cells in terms of antigen recognition suggests non-redundant, and perhaps complementary, functions of CD4+ and CD8+ T regulatory cells. This emphasizes the importance and necessity of continuous research on both subpopulations of regulatory T cells so as to decipher their complex physiological relevance and possible synergy in homeostasis and in clinical settings.

1.7 Current therapeutic approaches for HNSCC

The current therapeutic approaches for HNSCC include radical surgery and radiotherapy (RT), and for advanced stages either radical surgery followed by chemo/radiotherapy (CRT), primary chemo radiotherapy, combination of chemo/radiotherapy with Cetuximab (an anti-epidermal growth factor-receptor), or pembrolizumab (ananti-PD1 monoclonal antibody) for recurrent/progressive disease [42-43-44-45]

To date, platinum-based chemotherapy remains first line systemic therapy both in the curative and recurrent and/or metastatic (R/M) setting. The abnormal activation of the epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, is a key feature of epithelial cancers, including head and neck cancer. In general, its presence is associated with a poor clinical outcome.

The approval of the epidermal growth factor-receptor (EGFR) antagonist cetuximab introduced the first targeted therapy in HNSCC showing increased locoregional control when compared to RT alone and improved outcome when combined with chemotherapy. Interestingly, the immune system appears to affect treatment response in the context of RT/CRT. Accumulating data regarding the interplay between tumor/microenvironment and the immune contexture as well as mechanisms underlying immune-checkpoint pathway regulation, suggest that targeted therapies can promote anti-tumor immunity and mediate durable cancer regression. Radiotherapy increases the expression of EGFR in cancer cells, so the combination with Cetuximab *(Erbitux, ImClone Systems)*, an IgG1 monoclonal antibody against the ligand binding domain of EGFR, stimulates antibody-dependent cell-mediated cytotoxicity. [46]

Despite these options available for treatment, the mortality rates for patients with HNSCC are still very high (mainly in advanced/metastatic or recurrent disease).

1.8 Radiotherapy

An updated view on radiotherapy (RT) mechanisms of action against tumors considers the activation of anti-cancer immune response as a key event. RT is considered a local therapy, that acts by direct energy deposition within the tumor bed leading to DNA lesions and extensive cell death.

Local effects enhance a phenomenon also known as “in situ vaccination”. The radiation “unmasks” the tumor through Immunogenic Cell Death, in other words eliciting antigen release from dying cancer cell. The availability of tumor antigen and neoantigen can thereby reverse the evasion mechanism from the immune response, making cancer accessible to both the innate and adaptive immune systems. [54]

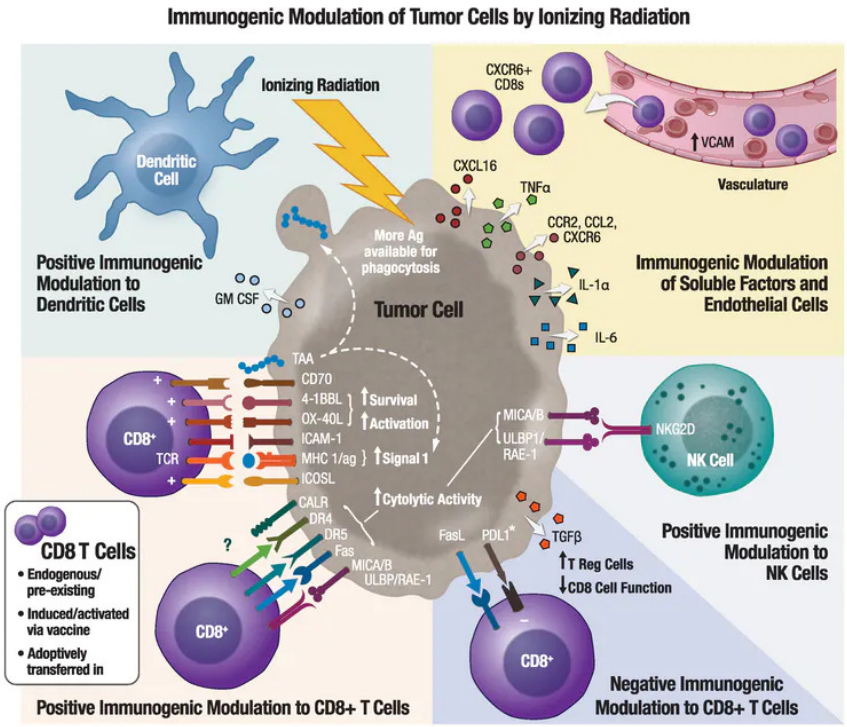

Fig.10 Pro-immunogenic and immunosuppressive properties of radiation.

Different and multiple mechanisms are involved in Immunogenic Cell Death induced by Radioterapy.

Irradiated cells release danger-associated molecular patterns (DAMPs), that act as extracellular signaling, such as:

-high mobility group box1 (HMGB1), heat-shock proteins, uric acid, activate toll-like receptor signaling, which leads to dendritic cell maturation and priming of cytotoxic T lymphocytes (CTLs);

-adenosine triphosphate (ATP) acts through cell surface purinergic receptors to attract macrophages and activate dendritic cells;

exposure of calreticulin on the tumor cell surface. Calreticulin acts as a phagocytotic signal for dendritic cells and macrophages.

Radiation also induces several phenotypic changes in tumor cells to facilitate immune detection. The recurrent expression of several cell surface death receptors enhances susceptibility of tumor cell death in the presence of immune cells expressing the corresponding ligands:

-upregulation of FAS receptor by radiation can be engaged by its specific ligand FAS-L to trigger extrinsic caspase-dependent apoptosis;

-radiation-induced death receptor 5 (DR5), that sensitizes cancer cells to apoptosis via binding of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL);

- expression of co-stimulatory molecule CD80 and stress-induced NKG2D ligand are promoted by radiation to facilitate tumor cell clearance by T cells and Natural Killer (NK) cells.

RT modulates the tumor vascular endothelium, inducing cell adhesion molecules that further promote recruitment of anti-tumor immune cells:

-increases the expression of E-selectin and ICAM1 in human endothelial cells thus modifying immune-cell permeability of the tumor vasculature,

-facilitates the recruitment of effector T-cells to tumors through the induction of chemokines. In particular, the recruitment of CD4+ and CD8+ T-cells is led by the induction of CXCL16, CXCL9 and CXCL10 chemokine.

Radiation also upregulates the release of pro-inflammatory cytokines, including interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF-α), and type I and type II interferons (IFNs).

Irradiated tumor cells upregulate MHC-I and facilitate tumor antigen uptake by DCs, in order to increase tumor detection by immune system, counteracting the escape phase (Fig.10) [**47- 48**-49-50-51]

With the induction of an Immunogenic Cell Death, that includes all these phenomena, radiotherapy engages both the innate and adaptive arms of the immune system, coupling the release of antigens with immune adjuvant-like effects, with the potential to convert the irradiated cancer into an in situ vaccine that elicits tumor-specific T cells. (Fig.11)

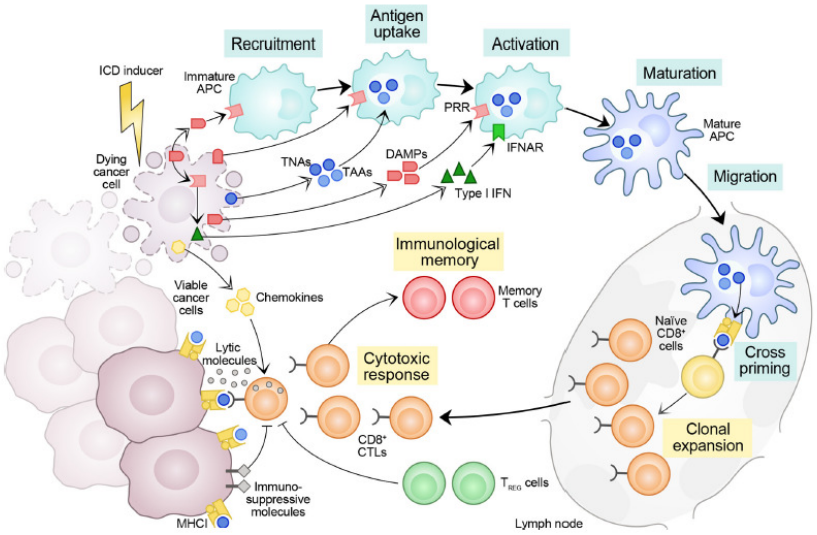

Fig.11 Cells undergoing immunogenic cell death in response to stress such as radiotherapy. Dying cells emit a panel of immunostimulatory damage-associated molecular patterns (DAMPs) and cytokines that support the recruitment, phagocytic activity and maturation of antigen-presenting cells (APCs). APC migrate to lymph nodes and prime a cytotoxic T lymphocyte (CTL)-dependent immune response and induce an immunological memory in periphery. [53]

It has also been recognized that local RT can act outside the directly irradiated site as well. RT systemic responses were observed towards tumor regression at sites distant from the irradiated region and is commonly known as the “abscopal effect”. RT effects synergize with other antitumor treatment modalities (chemotherapy, immunotherapy), and might influence long-term treatment outcome both in terms of local tumor control and development of distant metastases, initiating inflammatory and immune reactions outside the directly irradiated tissues. [52]

Despite a lot of data in literature supporting the systemic effect of radiotherapy, whose main function seems to be to act as an in-situ vaccination, little is known about the modulation of peripheral blood T cells population in response to this kind of therapy.

In Non-small Cell Lung cancer RT increased the frequency of total T cells, especially the proportion of CD8+ T cells, but decreased the frequency of inhibitory Tregs. Peripheral CD8+ and CD4+ T cells were mainly activated, expressing high levels of TNF-α, IFN-γ, granzyme B and IL-2. Also, PD-1 and Ki67 expression were found increased indicative of an active T-cell functionality. [56-57]

Almost the same effects were detected on Esophageal Cancer and Cervical Cancer patients accompanied by unfavorable CD8+ cells/Treg ratio upon radiation treatment. [58-59]

It has also been reported that in peripheral blood samples of HNSCC patients collected after radiotherapy, the frequency of Treg cells was almost two times higher than in healthy subjects; moreover, CTLA4-expressing CD4 cells and PD-1 expressing T effector cells were significantly higher in patients before treatment than in controls. In this paper was also observed that the fraction of proliferating CD4+ Treg cells increased after RT and that the levels of CTLA4+ and PD-1+ increased on CD4+ T effector cells. [60]

2. AIM

The purpose of this study was the immunological monitoring through multicolor flow cytometry of circulating T cells in HNSCC patients, analyzed before and after chemo-radiotherapy treatment, in order to define the quality and intensity of the peripheral immunological response.

Hence, multiparametric flow cytometry panels were structured to delineate :

- CD4+ and CD8+ T lymphocyte maturation stages;
- the cytokine production, such as IFN-γ, IL-17, IL-10 and Granzime B by CD4+ and CD8+ T lymphocytes;
- the regulatory commitment in both CD4+ Treg and CD8+ Treg ;
- the expression of markers of exhaustion/IRs (CD39, PD-1, CTLA-4) by T effector and T regulatory subsets.

3. Materials and Methods

3.1 Patients

This was a descriptive observational clinical study. Peripheral blood samples were collected from **20** patients affected by HNSCC (Table 3) who were enrolled at the Day-Hospital Therapeutics of the Radiotherapeutic Oncology Operative Unit and Otorhinolaryngology Clinic of the IRCCS Policlinico San Martino Hospital. The **20** patients enrolled were monitored up to 32 months (range from 18 to 32 months for alive patients) in order to determine their clinical outcome.

The study was carried out in compliance with the Helsinki Declaration and approved by the Ethical Committee of the San Martino Hospital in Genoa (P.R.133REG2017). All enrolled patients provided written informed consent.

3.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by centrifugation on Ficoll-Hypaque density gradient (Biochrom AG). PBMCs were collected and washed twice with 1X PBS phosphate buffer saline and resuspended in RPMI 1640 (Invitrogen) supplemented with 10% autologous plasma. The cells were counted in crystal violet and brought to a concentration of $10x10^7$/ ml.

3.3 Immunofluorescence analyses

Immunofluorescence analyses were performed on $1x10^6$ PMBCs in 100 µl of PBS incubating with specific fluorochrome-conjugated monoclonal antibodies (mAbs).

The following mAbs specific for surface markers were used (Supplementary Table 2): Brilliant Violet(BV)605-conjugated anti-human CD45RA clone HI100 (BD Biosciences), phycoerythrin (PE) or cyanin (Cy) 7-conjugated anti-human CCR7 clone 3D12 ((BD Biosciences), phycoerythrin (PE)-conjugated or BV421-conjugated anti-human CD127 clone HIL-7R-M21 (BD Biosciences), Peridinin Chlorophyll Protein Complex-cyanin 5.5 (PerCP-Cy5.5)-conjugated or PE-Cy5.5-conjugated anti-human CD28 clone CD28.2 (BD Biosciences), fluorescein isothiocyanate (FITC)-conjugated anti-human CD25 clone M-A251 (BD Biosciences), BV711-conjugated anti-CD39 clone TU66 (BD Biosciences), BV650-conjugated anti-human TIM-3 clone 7D3 (BD Biosciences), BV786-conjugated anti-human PD-1 clone MIH4 (BD Biosciences), allophycocyanin (APC)-conjugated anti-human CD152 (CTLA-4) clone BNI3 (BD Biosciences), APC-H7-conjugated anti-human CD4 clone RPA-T4 (BD Biosciences), BV 421-conjugated or or Alexa Fluor 647-conjugated or PerCP-Cy5.5-conjugated anti-human CD8 clone RPA-T8 (BD Biosciences), BV450-conjugated or Alexa Fluor 700-conjugated anti-human CD3 clone UCHT1 (BD Biosciences). To exclude dead cells, the samples were incubated with Aqua dead (Molecular Probes, Thermo Fisher) for 15 min at room temperature, before proceeding with surface staining. Cells were washed with PBS-BSA 0.01% and incubated with the surface mAbs.

After surface staining, the cells were fixed and permeabilized by Transcription Buffer Set (BD Pharmingen) prior to perform intranuclear staining for 30 min in the dark with the following mAbs: PE-CF594-conjugated anti-human FoxP3 clone 259D/C7 (BD Biosciences). The cells were washed with 1 ml of PBS-BSA 0.01% and resuspended in 300 µl of PBS. The samples were acquired by a BD Fortessa X20 flow cytometer (BD Biosciences) using the BD FACS Diva™ software version 8.0 (BD Biosciences), and analyzed using FlowJo™ software v10.7.

3.4 Analysis of intracellular cytokines by flow cytometry

The intracellular production of IFN-γ, IL-17A, IL-10 and GranzimeB by T lymphocytes from PBMC was analyzed as follows: the cells (resuspended in culture medium conditioned with 10% autologous plasma at a concentration of 1 x 10^7/ml) were stimulated with phorbol-12-myristate-13-acetate (PMA 50 ng/ml, Sigma) and ionomycin (2 µg/ml, Sigma) for 5 hours at 37°C. Brefeldin A (BFA 10 µg/ml, Sigma) was added to the cells for the last 4 hours of incubation. After washings, the samples were stained with fluorochrome-conjugated mAbs specific for surface markers [PerCPCy5.5 anti-CD3 (BD), APC-Cy7 anti-CD8 (e-Biosciences) and Violet Live/Dead Fixable Dead Cell stain (Life Technologies, CA, USA)], before fixing and permeabilizing the lymphocytes with the Cytofix/Cytoperm kit (BD Bioscience) following the manufacturer's instructions. The cells were washed in Perm-Wash buffer (BD Bioscience) and incubated with Pe-Cy7 anti-IFNγ (BD), FITC anti- GranzimeB (BD), PE anti-IL17A (e-Bioscience) mAbs, PECF594 anti IL10 (BD). Thereafter, the samples were washed in Perm-Wash buffer, fixed with FACS Lysing solution (BD Bioscience) and stored at 4 °C. The cytokine profile was acquired by a BD Fortessa X20 flow cytometer (BD Biosciences) using the BD FACS Diva™ software version 8.0 (BD Biosciences), and analyzed using FlowJo™ software v10.7.

3.5 Statistical analyses

Results were reported with mean and standard deviation (SD). To compare the frequency of the different T cell subsets between the tumor infiltrates of the two groups, we used the independent samples Student's t-test for not skewed populations or non-parametric Mann-Whitney test for all other populations; two-sided tests were always applied.

Analyses were performed by GraphPad Prism 5 for Mac OS X and Stata Statistical Software, Release 16. StataCorp LLC**.**

4. Results

To perform the project, circulating CD4+ and CD8+ T lymphocyte sub-populations, with effector or regulatory characteristics, were analyzed in 20 HNSCC patients before and after undergoing chemo-radiotherapy treatment.

HNSCC patients have distinct characteristics, summarized in Table 1, in terms of: HPV infection (9 HPV negative tumors, 10 HPV-16-associated HNSCC), smoking and tumor staging.

Blood samples collection acquisition by flow cytometry was conducted prior to initiation of anticancer treatment, and after a period of 1 month from the interruption of the anticancer treatment.

	Smoke	HPV-16	Stage	Therapy
Subject 1	Yes	Negative	4b2b	RT+CHT
Subject 2	Yes	Negative	4a2b	RT+CHT
Subject 3	Yes	Positive	4b2c	RT+CHT
Subject 4	Yes	Positive	4N1	RT+CHT
Subject 5	Yes	Negative	42c	RT+CHT
Subject 6	Yes	Negative	2n0	RT
Subject 7	Yes	Negative	1n2b	RT+CHT
Subject 8	Yes	Positive	4n2c	RT+CHT
Subject 9	Yes	Negative	4an2b	RT+CHT
Subject 10	Yes	Positive	4n2c	RT+CHT
Subject 11	Yes	Negative	2n2c	RT+CHT
Subject 12	No	Positive	4n2c	RT+CHT
Subject 13	No	Positive	na	Surgery
Subject 14	yes	Negative	4n3	RT-CHT
Subject 15	No	Positive	2n2b	RT+CHT
Subject 16	No	Positive	2n0	RT
Subject 17	Yes	Positive	1n2c	RT+CHT
Subject 18	No	Positive	2n1	RT
Subject 19	Yes	n.a.	4n2c	RT+CHT
Subject 20	Yes	Negative	4an2c	RT+CHT

Table 1: Pathological features of patients enrolled in the study.

4.1 CD4+ and CD8+ T lymphocyte populations

CD4+ and CD8+ T lymphocyte subpopulations have been analyzed by flow cytometry in Peripheral Blood Mononuclear Cells (PBMC) isolated from blood samples collected from patients before therapy (PRE) and after therapy (POST), and compared with PBMC derived from healthy controls (HC, n = 9), matched for age and sex. Fig 12 A shows the gating strategy that includes: 1) Discrimination of viable CD3 + cells (CD45 dot plot versus viability dye); 2) Discrimination of the lymphocyte population by light scatter characteristics (dot plot FSC-A versus SSC-A); 3) Electronic exclusion of Singlets doublets (FSC-A versus FSC-H dot plot); 4) Definition of the CD4 + and CD8 + T populations (CD4 versus CD8 dot plot).

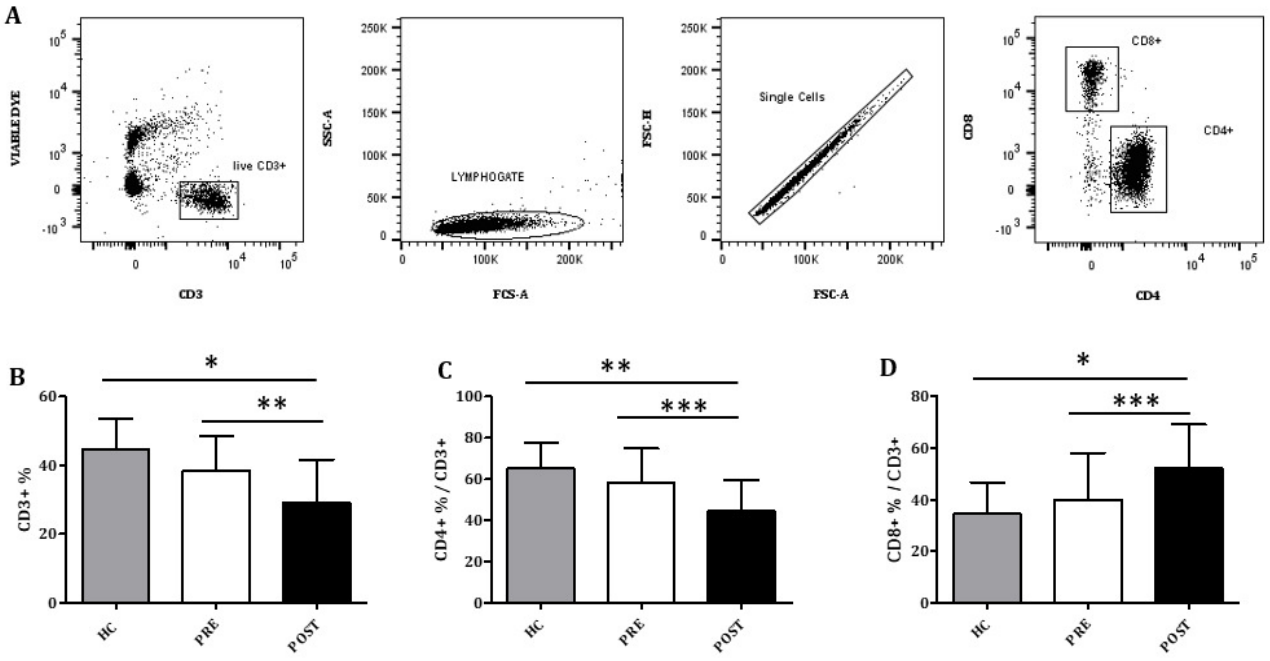

Fig.12 T-cell subsets in PBMC of HNSCC patients before and after therapy and PBMC of healthy controls.
A. Dot plots show the gating strategy of multiparametric flow cytometry analysis utilized for the selection of the CD3+CD4+ and CD3+CD8+ T cell subpopulations. **B.** Histograms show the percentage of total CD3+ T cells of PBMC in healthy controls (HC), in PBMC collected from HNSCC patients before (PRE) and after therapy (POST). **C. and D.** Histograms show the percentage of CD4+ and CD8+ T cells of PBMC in healty controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST), referred to CD3+ cells. Values are represented as Mean ± SD; Statistical significance was determined using paired or unpaired Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

CD3+ T lymphocyte frequency of PBMC POST samples resulted significantly lower to those of both PBMC PRE samples (28.9 ± 12 % versus 38.3 ± 10 , $p < 0.001$) and control subjects (44.6 ± 9%, $p<0.05$). (Fig 12 B)

The same behavior was observed regarding the frequency of circulating CD4+ T subset that significantly decreased after therapy (58 ± 16% versus 44 ± 15%; $p < 0.0001$), and resulted lower in POST samples compared to HC (65 ± 12%). (Fig 12 C)

Whereas, CD8+ T cell population showed an increment in samples collected from treated patients: in fact, the data showed a significant difference between PBMC POST and both PBMC PRE (52 ± 17% versus 44 ± 18%; $p < 0.0001$) and PBMC HC (34 ± 12%, $p<0.001$). (Fig 12 D)

4.2 Non-regulatory CD4+ T cells: maturation and cytokine production

To describe the maturation stages of CD4+T lymphocyte, CD45RA and CCR7 markers were used. As shown in figure 13 Panel A**,** the use of these two markers allows to identify four different CD4+ lymphocytes maturation stages: NAIVE (CD45RA+CCR7+), Central Memory T cells (CM; CD45RA-CCR7+), Effector Memory (EM; CD45RA-CCR7-) and Terminal Effector Memory (TEMRA; CD45RA+CCR7-).

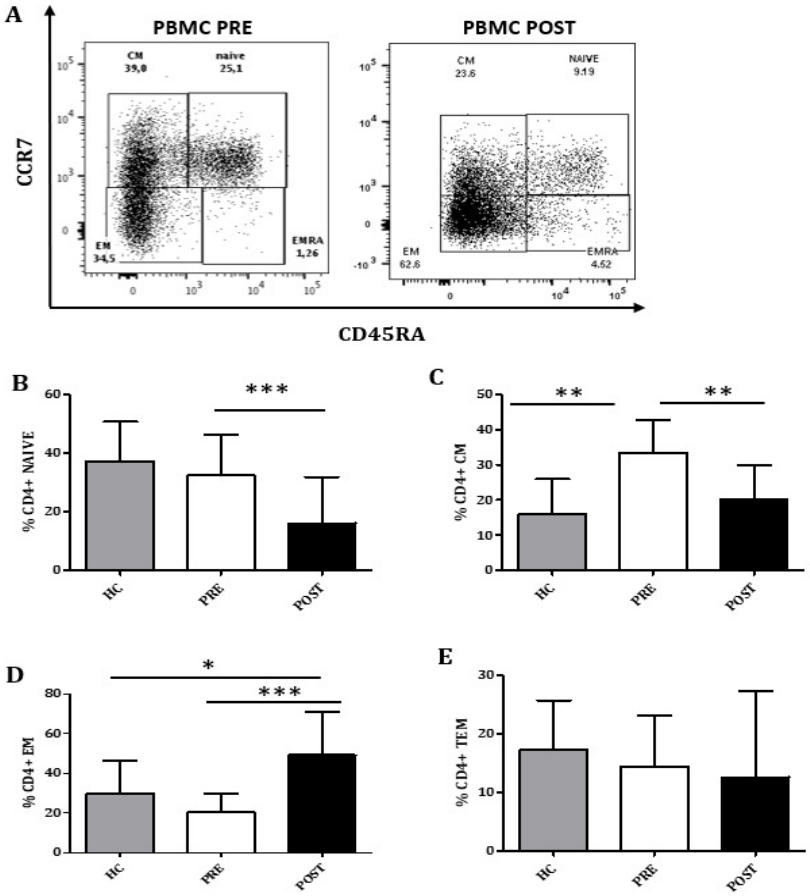

Fig.13 CD4+ T cells maturation in PBMC of HNSCC patients before and after therapy and PBMC of healthy controls. **A.** Dot plots show the maturation of CD3+CD4+ along the expression of CD45RA and CCR7 markers: T Naïve (CD45RA+CCR7+) Central memory T cells or CM (CD45RA-CCR7+) T Effector Memory or EM (CD45RA- CCR7-)Terminal Effector Memory or TEMRA (CD45RA+ CCR7-). **B. C. D. and E.** Histograms show the percentage of CD3+CD4+ Naïve cells, CD3+CD4+ CM cells, CD3+CD4+ EM cells, CD3+CD4+ TEMRA cells respectively of PBMC in healty controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). Values are represented as Mean ± SD; Statistical significance was determined using paired or unpaired Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

In the cohort of samples examined, CD4+ Naïve T cells constituted the 37± 13% of T cells in the peripheral blood of healthy controls, their percentage in PBMC PRE was 32.4 ± 13% and in PBMC POST was 16±15% (Fig 13 B). The percentage in samples collected after therapy was significantly decreased compared to PBMC PRE ($p < 0.0001$).

CM CD4+ T cells (Fig 13 C) were significantly less present among PBMC HC population (16 ± 9%) compared to CM subset in PBMC PRE (33 ± 9%; $p < 0.005$); CM CD4+ T population was significantly lower among PBMC POST (20 ± 9%; $p < 0.005$) compared with samples collected from patients HNSCC before therapy.

CD4+ Effector Memory T cells in PBMC HC and in PBMC PRE were comparable (29.6 ± 16% vs. 20 ± 9%). They significantly increased in PBMC POST compared to PBMC PRE (49 ± 9%; $p < 0.0001$) and to PBMC HC ($p<0.05$). (Fig 13 D)

Percentages of Terminal Effector memory CD4+ T cells in samples derived from patients at different time point and control donors were comparable (PBMC HC 17± 8%; PBMC PRE 14.4 ± 8%; PBMC POST 12.6 ± 14%). (Fig 13 E)

To evaluate if the therapy could influence the effector capacities of CD4 + T cells in terms of cytokine release and cytotoxicity, the intracellular production of IFN-γ, IL1-7A, IL-10 and Granzime B by CD4+ T lymphocytes was taken into consideration before and after therapy. The data showed no significant difference in the production of IFN-γ, IL-17A or IL-10 cytokines between the CD4+ T lymphocytes of the PMBC PRE and PBMC POST (data not shown).

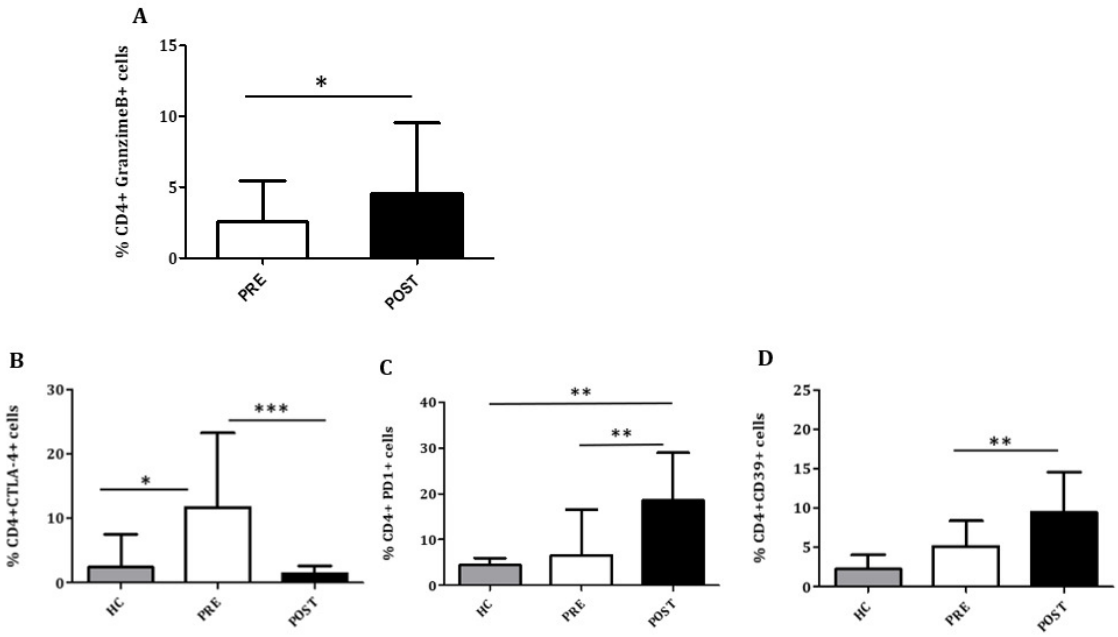

Fig.14 Frequencies of CD4+ T effector cells.
A. and B. Histograms show the percentage of CD4+GranzimeB+ T lymphocytes in PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). **C. D. and E.** Histograms show the percentage CD4+CTLA-4+, CD4+PD1+ and CD4+CD39+ T lymphocytes respectively of PBMC in healthy controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). Values are represented as Mean ± SD; Statistical significance was determined using paired or unpaired Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

Whereas the analyses showed a significant increment of the percentage of Granzime B+ CD4+T cells in blood samples collected after therapy (PBMC PRE 2.5± 2.9% vs PBMC POST 4.2 ± 4.9%; $p < 0.05$). (Fig 14 A)

Moreover, we examined the impact of anticancer therapy on expression of Inhibitory receptors (iRs)/exhaustion markers, as CTLA-4, PD-1 and CD39, on peripheral CD4+T cells.

In general, the expression of CTLA-4, PD-1 and CD39 exhaustion markers resulted higher on CD4+T cells in samples derived before the therapy than in samples derived from healthy controls, as expected in a chronic immune stress situation.

The data highlighted that the percentage of CTLA-4+ CD4+ T cells was significantly higher in PBMC PRE compared to that in both PBMC HC (11.6 ± 11.5% vs 2.3 ± 5.1%, respectively; $p < 0.05$) and PBMC POST (1.4 ± 1.6%; $p < 0.0001$). (Figure 14 B)

The analyses of PD-1+CD4+ T cells showed increased frequency of these cells in PBMC POST (18.5 ± 10.4 %) with respect to that in both PBMC PRE (6.5 ± 9.9%, $p < 0.001$) and PBMC HC (4.3 ± 1.5%, $p < 0.001$). (Figure 14 C)

Similar findings were observed concerning CD39 expression. In fact, the frequency of CD39+ CD4+ T-cell subset was increased in the of PBMC POST (9.9 ± 5.1%) compared to those of both PBMC PRE (18.0 ± 11.2%; $p < 0.001$) and PBMC HC (2.2 ± 1.8%) (Figure 14 D)

4.3 CD4+ Treg

Figure 15 A shows the gating strategy for the evaluation of the CD4+CD25^{high} FOXP3+ Treg population; the CD25^{high} FOXP3+ population was defined on the CD3+CD4+ population (dot plot analyzing FoxP3 versus CD25) and the CD25^{high} FOXP3+ gate was verified for negativity on the CD3+CD8+ population.

In general, the frequency of CD4+CD25^{high} FOXP3+ regulatory T cells resulted higher in the peripheral blood of HNSCC subjects before and after therapy (PBMC PRE 2.02 ± 0.9%; PBMC POST 1.8 ± 1.5%;) compared to healthy controls (PBMC HC 0.7 ± 0.28%) even without reaching statistical significance. (Figure 15 B)

Attention was also posed on a functional characterization of CD4+ Treg.

Based on what is proposed by the model described by Sakaguchi, we can distinguish three subpopulations, equipped or not with suppressive activity, using the modulation of FoxP3/CD25 and CD45RA markers :

(i) Naïve or resting Treg cells (CD45RA+FoxP3^{low} /CD25^{low}) with low suppressive activity; (ii) effector or activated Treg cells (CD45RA–FoxP3^{high}/CD25^{high}) with strong suppressive function; (iii) non-Treg cells (CD45RA– FoxP3^{low}/CD25^{low}) without suppressive ability .

The frequency of Naïve Treg was higher among PBMC HC (13.6 ± 5.4%) compared to PBMC PRE (7.9 ± 5.2%) and PBMC POST (4.5 ± 4.1%) even without reaching statistical significance;

however, in patients Naïve Treg frequency was significantly decreased in PBMC POST with respect to PBMC PRE value ($p < 0.05$). (Figure 15 C)

Non-Treg CD45RA– FoxP3 ^{low}/CD25^{low} CD4+ T cells were more represented in PBMC PRE (31 ± 14.2%) than in both PBMC HC (18.5 ± 5.6%, difference not fully significant) and PBMC POST (15.3 ± 6.2 %; $p < 0.0001$). (Figure 15 D)

Consistent with the possible effect of the therapy, activated CD4+Treg significantly increased in post-therapy compared to pre-therapy samples (PBMC PRE 14± 7.3% vs PBMC POST 23.6 ± 8.5%; $p < 0.0001$). (Figure 15 E)

No significant differences among groups were observed concerning the frequencies of PD-1+ CD4+ Treg (Figure 15 F).

Instead the frequency of CD39+ Treg was significantly increased in PBMC POST (58.3 ± 23%) with respect to both PBMC PRE (47.8 ± 24%, $p<0.05$) and PBMC HC (20.4 ± 27%, $p<0.05$). (Figure 15 G)

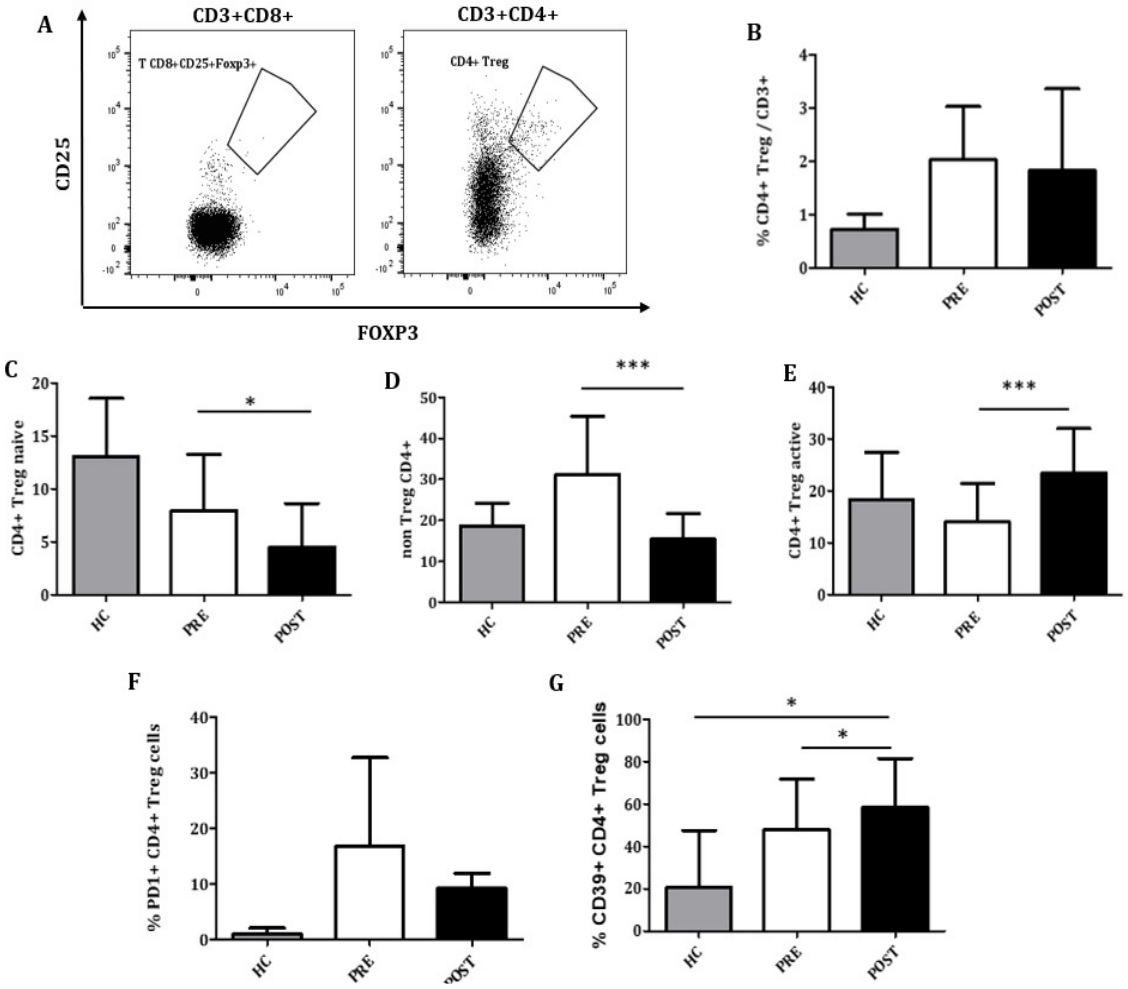

Fig. 15 CD4+ Treg cell subsets in PBMC of HNSCC patients before and after therapy and PBMC of healthy controls.
A. Dot plot representation shows the gating strategy of multiparametric flow cytometry analysis utilized for the selection of the CD4+ Treg cells population. **B.** Histograms show the percentage of CD4+ Treg cells of PBMC in healthy controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST), referred to CD3+ T cells **C. D. and E.** Histograms show the percentage of Naïve or resting Treg cell (CD45RA+FoxP3^{low}/CD25low), non-Treg cells (CD45RA– FoxP3^{low}/CD25low) and effector or activated Treg cells (CD45RA– FoxP3^{low}/CD25high) respectively of PBMC in healthy controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST), referred to CD3+ T cells. **F. and G** Histograms show the percentage CD4+ PD1+Treg and CD4+ CD39+Treg T lymphocytes respectively of PBMC in healthy controls (HC), of PBMC collected from HNSCC patients before (PRE) and after therapy (POST). Values are represented as Mean ± SD; Statistical significance was determined using paired or unpaired Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

4.4 Non-Treg CD8+ T cells: maturation and cytokine production

To describe the dynamic of CD8+T cells induced by anticancer therapy, the analysis of maturation stages was performed in peripheral CD8+ T lymphocytes, evaluating the expression of CD45RA and CCR7 markers to identify: NAIVE cells (CD45RA+CCR7+), Central Memory cells (CM; CD45RA-CCR7+), Effector Memory cells (EM; CD45RA-CCR7-) and Terminal Effector Memory cells (TEMRA; CD45RA+CCR7-), as shown in Figure 16, before and after anticancer therapy (Panel A) in peripheral blood derived from HNSCC patients and healthy controls.

A decreased frequency of Naïve CD8+ T cells was observed in PBMC POST (6.3 ± 9.4%) with respect to PBMC PRE (13.3 ± 13.7%, $p < 0.05$) and to PBMC HC (17.6± 8.7%, not significant). (Fig 16 B)

Concerning CM CD8+ T cell frequency, it was lower in less in PBMC POST (2 ± 1.4 %) than in PBMC PRE (3.4 ± 2.8%, $p < 0.001$) and PBMC HC (5.3 ± 2.8%, not significant) (Fig 16 C)

The frequencies of EM CD8+ T cells in PBMC PRE were significantly higher than in both PBMC POST (37.7 ± 30% vs. 18 ± 9.9%; $p < 0.001$) and PBMC HC (14.8 ± 12.9%, not significant). (Fig 16 D)

Finally, the frequency of TEM CD8+ T cells in PBMC PRE (44 ± 25.2%) resulted significantly lower than in PBMC POST (72.3 ± 13%, $p < 0.001$) and comparable to that of PBMC HC (63.8± 14.4%). (Figure 16 E)

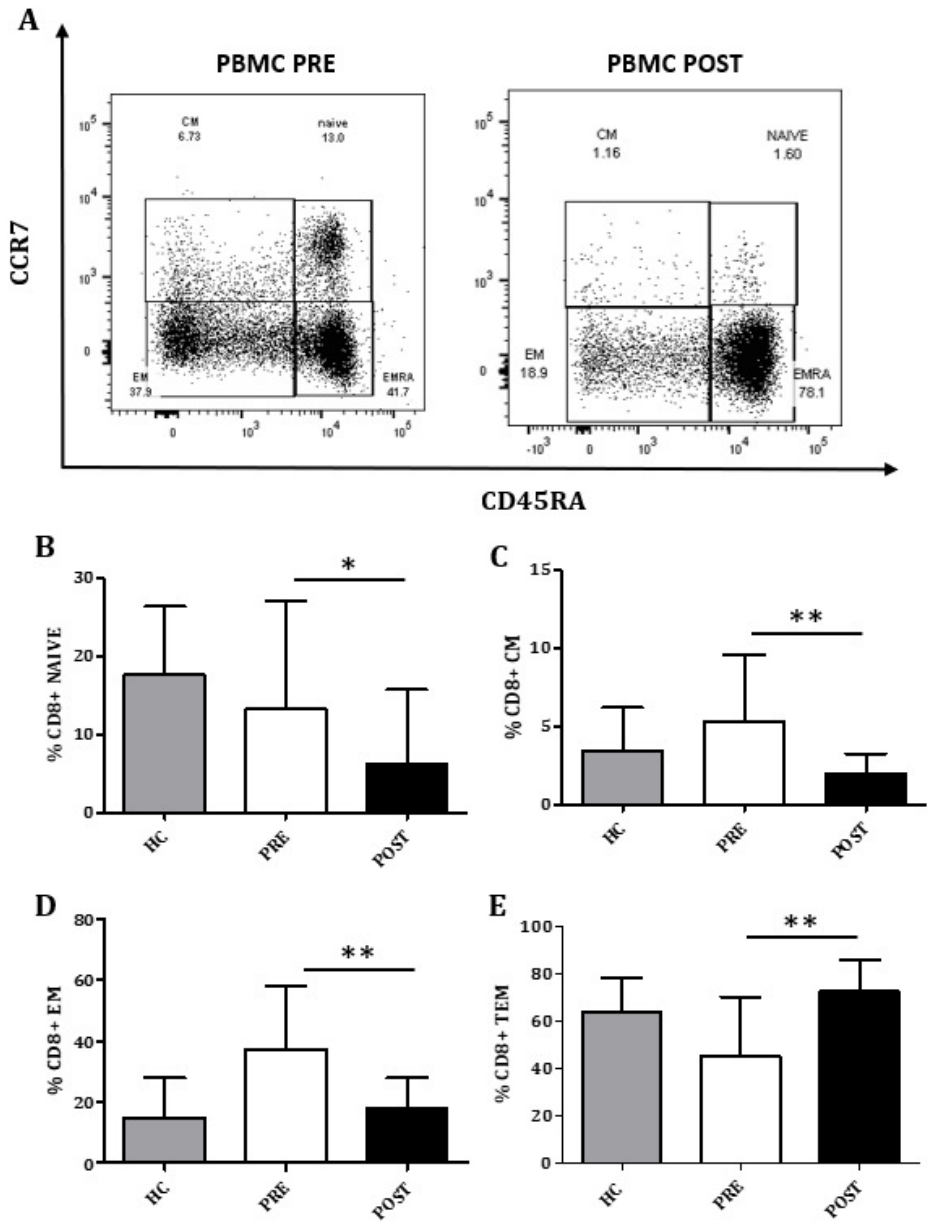

Fig.16 CD8+ T cells maturation in PBMC of HNSCC patients before and after therapy and PBMC of healthy controls. **A.** Dot plot representation show the maturation of CD3+CD8+ along the expression of CD45RA and CCR7 markers: T Naïve (CD45RA+CCR7+) Central memory T cells or CM (CD45RA-CCR7+) T Effector Memory or EM (CD45RA- CCR7-)Terminal Effector Memory or TEMRA (CD45RA+ CCR7-). **B. C. D. and E.** Histograms show the percentage of CD3+CD8+ Naïve cells, CD3+CD8+ CM cells, CD3+CD8+ EM cells, CD3+CD8+ TEMRA cells respectively of PBMC in healthy controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). Values are represented as Mean ± SD; Statistical significance was determined using paired or unpaired Student's t-test: *p<0.05; **p<0.01.

As for the CD4+ T cells, non-Treg CD8+T cells were also evaluated before and after anticancer therapy for: (i) intracellular production of IFN-γ, IL-17A, IL-10 cytokines and Granzime B by CD8+ T lymphocytes; (ii) expression of co-stimulatory (CD28) and co-inhibitory molecules (CTLA-4, PD-1 and CD39).

Concerning both cytokine profile and Granzyme production by non-Treg CD8+T cells, no significant differences in production of any analyzed cytokine (IL-17A, IL-10 and IFNγ+) were found among the different groups (data not shown).

Interestingly, the frequency of CD8+CD28- T cells resulted significantly higher in PBMC PRE if compared with samples collected from healthy subjects (PBMC HC) (50.3 ± 29.8% vs 24.2 ± 7.2%; $p < 0.05$) and further increased in PBMC POST (63.5 ± 22.8%; $p < 0.05$). (Fig 17 C)

Regarding the assessment of the state of exhaustion, the expression of Inhibitory receptors (iRs), as CTLA-4, PD-1 and CD39 was also examined on CD8+T cells.

The data showed:

i) comparable frequencies of both CTLA-4+CD8+ and CD39+CD8+ T cell subsets among the groups (Fig 16 D and Fig 17 F);

ii) significantly increased frequencies of PD-1+CD8+ T cells in PBMC POST with respect to PBMC HC (20.2 ± 14.4% cells vs 6.1 ± 3.3%, $p<0.01$). (Fig 17 E)

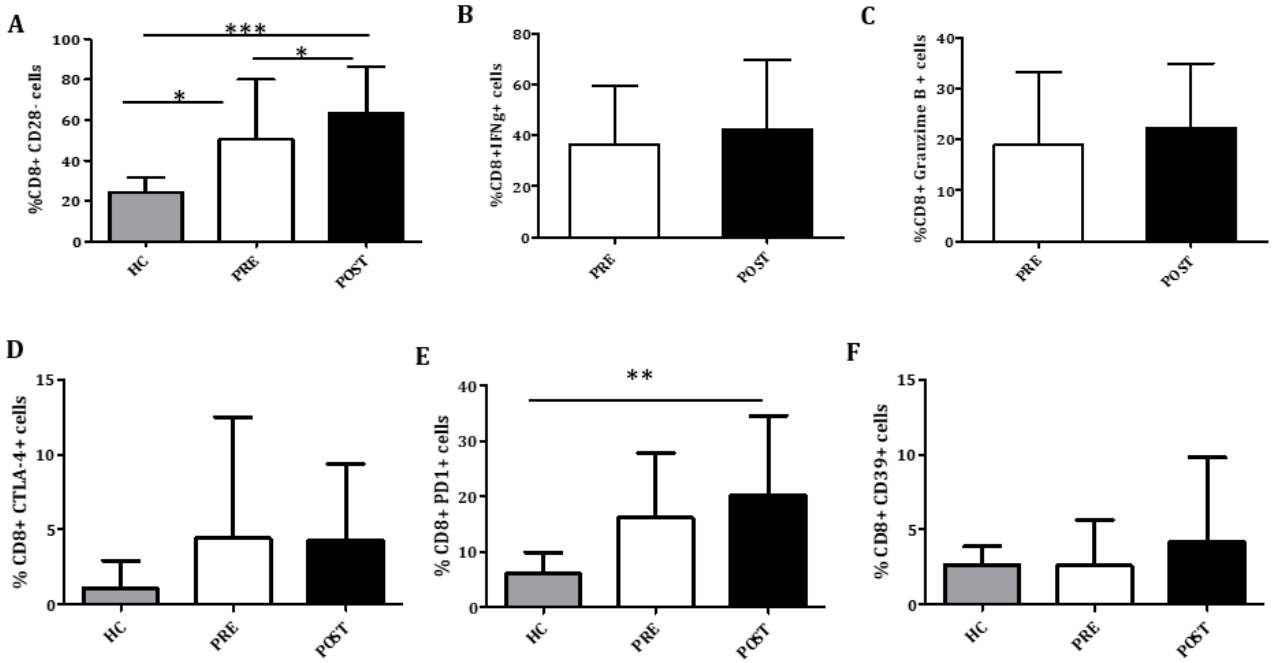

Fig.17 Frequencies of CD8+ T cells effector.

A. Histograms show the percentage CD8+CD28-+ lymphocytes respectively of PBMC in healthy controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). **B.** Histograms show the percentage of CD8+IFNγ+ and CD8+IL17+ T lymphocytes in PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). **D. E. and F.** Histograms show the percentage CD8+CTLA-4+, CD8+PD1+ and CD8+CD39+ T lymphocytes respectively of PBMC in healthy controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). Values are represented as Mean ± SD; Statistical significance was determined using paired or unpaired Student's t-test: *p<0.05; **p<0.01; ***p<0.001.

4.5 CD8+ Treg

Figure 18 A shows the gating strategy for the analysis of the CD8+ Treg population. Briefly, the CD8+ CD28- population (dot plot showing CD8 versus CD28) was initially defined by verifying the negativity threshold of the CD28 marker on the CD3+CD4+ T population (dot plot showing CD4 versus CD28). Then, the subset of CD127-CD39+ T cells (dot plot showing CD127 versus CD39) was identified on the CD8+CD28- T cell population, , thus defining the CD8+CD28-CD127-CD39+ T regulatory subset.

To fully analyze the arm of the regulatory T cells, the frequencies of CD8+CD28-CD127-CD39+ T regulatory lymphocytes were evaluated in healthy donors and in samples of HNSCC patients before and after anticancer therapy: no significant differences were detected among the three group.s (Fig 18 A).

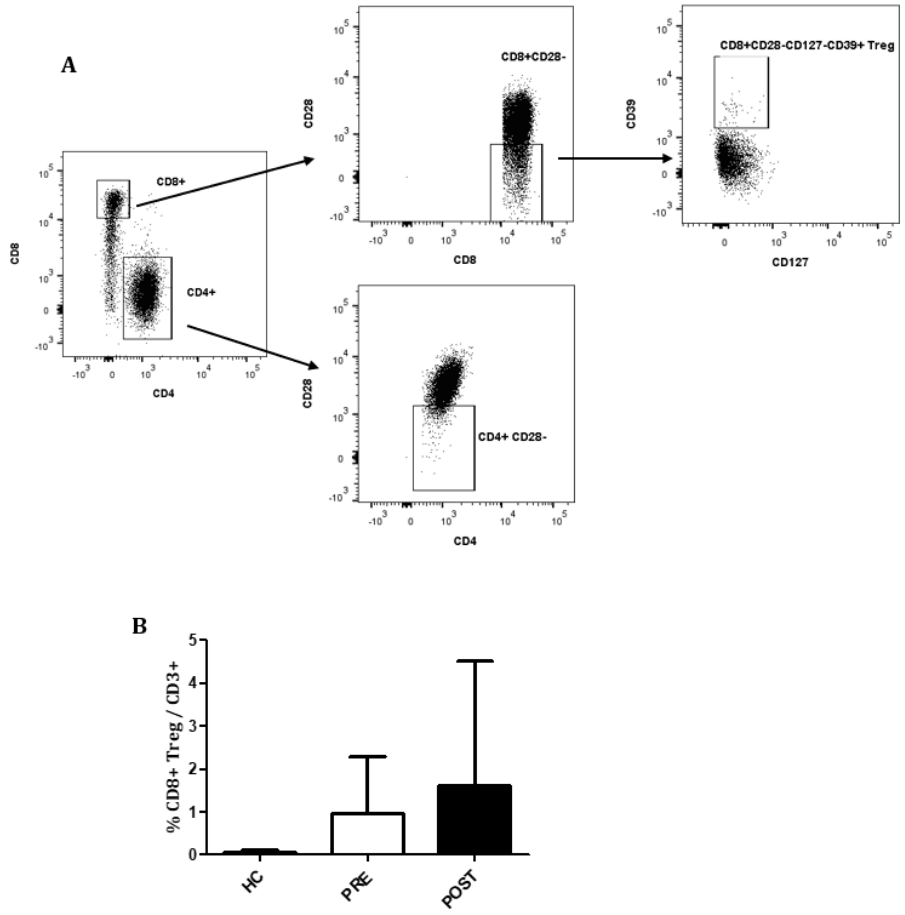

Fig.18 CD8+ Treg cell subsets in PBMC of HNSCC patients before and after therapy and PBMC of healthy controls. **A.** Dot plots show the gating strategy of multiparametric flow cytometry analysis utilized for the selection of the CD8+ Treg cells population. **B.** Histograms show the percentage of CD8+ Treg cells of PBMC in healty controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST), referred to CD3+ cells **C.** Histograms show the percentage CD8+ Treg PD1+ lymphocytes respectively of PBMC in healty controls (HC), of PBMC collected from HNSCC subjects before (PRE) and after therapy (POST). Values are represented as Mean ± SD.

5. Discussion

HNSCC is a relevant medical issue since it is the sixth most common cancer globally and represent about 3% of all malignant tumors in Italy. The malignant tumors of the head and neck are almost represented by squamous cell carcinomas with an extremely variable biological behavior.

HNSCC is generally associated with a history of alcohol and tobacco consumption or abuse, and an etiopathogenetic factor is HPV infection.

Surgery, radiotherapy and chemotherapy in various combinations are used in the treatment of head and neck cancers, depending on the TNM stage. Radiotherapy associated with cisplatin-based chemotherapy is considered the standard, as chemo-radiotherapy compared to radiotherapy alone has shown a significant improvement in overall survival.

Frequent data in the literature are focused on monitoring the immune response of patients undergoing chemotherapy, radiotherapy or immunotherapy treatments. Very few studies, especially on head and neck tumors, assessed so far the characteristics of the peripheral immune response in reference to the chemoradiotherapy treatment.

Balázs K. and collegues [60] reported that in peripheral blood samples of HNSCC patients collected after radiotherapy the fraction of Treg cells was almost two times higher than healthy controls, and CTLA4-expressing CD4 cells and PD-1 expressing T effector cells were significantly higher in patients before treatment with respect to healthy controls. They also observed that the fraction of proliferating CD4+ Treg cells increased after RT and that the levels of CTLA4+ and PD-1+ increased on CD4+ T effector cells.

Exposure of tissue to ionizing radiation and chemo agents has different effects from inflammation to distinct forms of programmed cell death. It is known that the success of concurrent chemo-radiation treatment relies in the achievement of immunogenic cell death and in the consequent release of tumor neoantigens in an immunogenic way. On the other hand, is well known the adverse influence of chemo/radiotherapy on affecting leukocyte's production by the bone marrow, that could lead to abnormal hematopoiesis, in addition to a significant reduction in the number of the leukocytes that could persist also 4 weeks after treatment.

Immunogenic cell death (ICD) and priming of antitumor T-cell responses are closely related, in fact ICD is an efficient process to transfer antigens from tumor cells to DCs and that DCs are required to activate tumor-specific T cells. Different signals are required to achieve an ICD , they include: 1) cell surface translocation of calreticulin (an endoplasmic reticulum resident protein); 2) extracellular release of high-mobility group protein B1 (HMGB1, a nonhistone nuclear protein), and 3) release of ATP (the primary unit of cellular energy transfer). These molecular signals are correlated to the instauration of a proinflammatory milieu, with the release of: 1) chemokines CXCL9, CXCL10, and CXCL16, which promote recruitment of effector CD8 and T-helper 1 CD4 T cells; 2) and proinflammatory cytokines induced by radiation including interleukin 1β, tumor necrosis factor α and type 1 and 2 interferons. In addition, tumor cells undergo phenotypic changes that enhance their susceptibility to immune effectors, enhancing the expression of death receptors, MHC class 1 molecules, costimulatory molecules, adhesion molecules, and stress-induced ligands, increasing their recognition and killing by T cells.

Chemo-radiotherapy treatment engages both the innate and adaptive arms of the immune system, by coupling release and/or expression of new antigens with immune adjuvant-like effects, and acts as a vaccine that elicits tumor-specific T cells. Once vaccinated, the host is endowed with immune memory, a powerful weapon active against synchronous non-irradiated tumor sites (abscopal effects) and potentially against cancer cells that emerge from dormancy during the life of the host.

In the study presented here, peripheral blood of 20 patients suffering for HNSCC were examined in order to define the qualitative and quantitative characteristics of their immunological T cell response, before and after these subjects underwent chemo-radiotherapy treatment using multiparametric flow cytometry.

In the cohort of patients here examined, it is evident a decrease of the CD3+ total T cell compartment after therapy. Concerning the CD4+ T cells, therapy mainly induced a switch from the naïve to the EM cell subset, associated with an increase in Granzyme secreting CD4+ T cells. These findings suggest that the treatment induced functional maturation of CD4+ T lymphocytes producing cells prone to exert effector functions. This is corroborated by the decrease of the CD4+ T cells expressing CTLA-4, that is an IR mainly expressed during early maturation stages, and by the increased frequency of CD4+ T cells expressing the PD-1 molecule, typical of late maturation stages, phenotypic changes shared by both non-Treg and Treg CD4+ T cell subsets.

Interestingly, the analysis of the CD8+ T cell compartment provided data supporting the concept that therapy may induce pro-maturation effects on T cells pushing them toward acquisition of full effector functions. Indeed, this is suggested by the expansion of the

CD8+CD28- T cell subset and by the decrease of the early maturation stages associated with the increase of TEM CD8+ T cells, a subset of effector T lymphocytes prone to migrate into tissues. Moreover, no signs of CD8+ Treg expansion were observed.

All together these data have relevance since apparently overturn the common view assessing for a hampering effect of ablative therapy on the immune system, instead suggesting its activatory effect on the immune system. This may find an explanation thinking at the huge amount of cell death products acting as danger signals raised by chemotherapy-Rt combination and by the subsequent stimulation of cancer specific immune responses induced by antigen spreading. The fact that both effector CD4+ and CD8+ T cell subsets, expanded after therapy, uploaded PD-1 is also noteworthy since supports the likely utility of a combined use of PD-1 blockers with chemo-Rt therapy.

In conclusion, our data show that in HNSCC patients current therapeutic approaches, based on administration of chemotherapy and/or radiotherapy, induce activation of the immune system leading to maturation of effector T cell subpopulations. Subsequent investigations on the leukocyte formula will prove useful and correlated with the more well-known effects of chemo-Rt therapy. Moreover they are in support of the inclusion of PD-1 inhibiting agents to the therapeutic armamentarium against HNSCC in order to prevent T cell exhaustion and to preserve their effector commitment.

References

1. B. A. Van Dijk, G. Gatta, R. Capocaccia, D. Pierannunzio, P. Strojan, L. Licitra, and R. W. Group, "Rare cancers of the head and neck area in Europe," Eur J Cancer, vol. 48, no. 6, pp. 783-96, 2012.
2. A. K. Chaturvedi, E. A. Engels, R. M. Pfeiffer, B. Y. Hernandez, W. Xiao, E. Kim, B. Jiang, M. T. Goodman, M. Sibug-Saber, W. Cozen, L. Liu, C. F. Lynch, N. Wentzensen, R. C. Jordan, S. Altekruse, W. F. Anderson, P. S. Rosenberg, and M. L. Gillison, "Human papillomavirus and rising oropharyngeal cancer incidence in the United States," J Clin Oncol, vol. 29, no. 32, pp. 4294-301, 2011.
3. K. B. Pytynia, K. R. Dahlstrom, and E. M. Sturgis, "Epidemiology of HPV-associated oropharyngeal cancer," *Oral Oncol,* vol. 50, no. 5, pp. 380-6, 2014.
4. G. D'Souza, A. R. Kreimer, R. Viscidi, M. Pawlita, C. Fakhry, W. M. Koch, W. H. Westra, and M. L. Gillison, "Case-control study of human papillomavirus and oropharyngeal cancer," N Engl J Med, vol. 356, no. 19, pp. 1944-56, 2007.
5. G. Calais, E. Bardet, C. Sire, M. Alfonsi, J. Bourhis, B. Rhein, J. Tortochaux, Y. T. Man, H. Auvray, and P. Garaud, "Radiotherapy with concomitant weekly docetaxel for Stages III/IV oropharynx carcinoma. Results of the 98-02 GORTEC Phase II trial," Int J Radiat Oncol Biol Phys, vol. 58, no. 1, pp. 161-6, 2004.
6. F. Denis, P. Garaud, E. Bardet, M. Alfonsi, C. Sire, T. Germain, P. Bergerot, B. Rhein, J. Tortochaux, and G. Calais, "Final results of the 94-01 French Head and Neck Oncology and Radiotherapy Group randomized trial comparing radiotherapy alone with

concomitant radiochemotherapy in advanced-stage oropharynx carcinoma," J Clin Oncol, vol. 22, no. 1, pp. 69-76, 2004.

7. Kuryk L, Møller AW, Garofalo M, et al. Antitumor-specific T-cell responses induced by oncolytic adenovirus ONCOS-102 (AdV5/3-D24-GM-CSF) in peritoneal mesothelioma mouse model. J Med Virol. 2018
8. Dirnhofer S, Zippelius A. Cancer immunology, inflammation, and tolerance: an introduction. Virchows Arch. 2019
9. Street MD, Doan T, Herd KA, Tindle RW. Limitations of HLA-transgenic mice in presentation of HLA-restricted cytotoxic T-cell epitopes from endogenously processed human papillomavirus type 16 E7 protein. Immunology. 2002
10. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331(6024):1565-1570. doi:10.1126/science.1203486
11. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity. 2004;21(2):137-148. doi:10.1016/j.immuni.2004.07.017
12. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1-10. doi:10.1016/j.immuni.2013.07.012
13. Janeway's Immunobiology (9th Edition)
14. Hall BM. T Cells: Soldiers and Spies--The Surveillance and Control of Effector T Cells by Regulatory T Cells. Clin J Am Soc Nephrol. 2015 Nov 6;10(11):2050-64. doi: 10.2215/CJN.06620714. Epub 2015 Apr 15. PMID: 25876770; PMCID: PMC4633791.

15. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999 Oct 14;401(6754):708-12. doi: 10.1038/44385. PMID: 10537110
16. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med. 2002 Apr;8(4):379-85. doi: 10.1038/nm0402-379. PMID: 11927944
17. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A. 2008 Nov;73(11):975-83. doi: 10.1002/cyto.a.20643. PMID: 18785267.
18. Zhu Y, Yao S, Chen L. Cell surface signaling molecules in the control of immune responses: a tide model. Immunity. 2011 Apr 22;34(4):466-78. doi: 10.1016/j.immuni.2011.04.008. PMID: 21511182; PMCID: PMC3176719.
19. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol. 2013 Apr;13(4):227-42. doi: 10.1038/nri3405. Epub 2013 Mar 8. Erratum in: Nat Rev Immunol. 2013 Jul;13(7):542. PMID: 23470321; PMCID: PMC3786574.
20. Fuertes Marraco SA, Neubert NJ, Verdeil G, Speiser DE. Inhibitory Receptors Beyond T Cell Exhaustion. Front Immunol. 2015 Jun 26;6:310. doi: 10.3389/fimmu.2015.00310. PMID: 26167163; PMCID: PMC4481276.

21. Zhu Y, Yao S, Chen L. Cell surface signaling molecules in the control of immune responses: a tide model. Immunity. 2011 Apr 22;34(4):466-78. doi: 10.1016/j.immuni.2011.04.008. PMID: 21511182; PMCID: PMC3176719.
22. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. J Immunol. 2012 Apr 1;188(7):2957-65. doi: 10.4049/jimmunol.1100038. PMID: 22442493; PMCID: PMC3320038.
23. Allard B, Longhi MS, Robson SC, Stagg J. The ectonucleotidases CD39 and CD73: Novel checkpoint inhibitor targets. Immunol Rev. 2017 Mar;276(1):121-144. doi: 10.1111/imr.12528. PMID: 28258700; PMCID: PMC5338647.
24. Bastid J, Regairaz A, Bonnefoy N, Déjou C, Giustiniani J, Laheurte C, Cochaud S, Laprevotte E, Funck-Brentano E, Hemon P, Gros L, Bec N, Larroque C, Alberici G, Bensussan A, Eliaou JF. Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. Cancer Immunol Res. 2015 Mar;3(3):254-65. doi: 10.1158/2326-6066.CIR-14-0018. Epub 2014 Nov 17. PMID: 25403716.
25. Antonioli L, Pacher P, Vizi ES, Haskó G. CD39 and CD73 in immunity and inflammation. Trends Mol Med. 2013 Jun;19(6):355-67. doi: 10.1016/j.molmed.2013.03.005. Epub 2013 Apr 17. PMID: 23601906; PMCID: PMC3674206.
26. Wherry EJ. T cell exhaustion. Nat Immunol. 2011 Jun;12(6):492-9. doi: 10.1038/ni.2035. PMID: 21739672
27. Catakovic K, Klieser E, Neureiter D, Geisberger R. T cell exhaustion: from pathophysiological basics to tumor immunotherapy. Cell Commun Signal. 2017 Jan

5;15(1):1. doi: 10.1186/s12964-016-0160-z. PMID: 28073373; PMCID: PMC5225559.

28. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 2015 Apr;36(4):265-76. doi: 10.1016/j.it.2015.02.008. Epub 2015 Mar 18. PMID: 25797516; PMCID: PMC4393798.
29. D. A. Vignali, L. W. Collison, and C. J. Workman, "How regulatory T cells work," Nat Rev Immunol, vol. 8, no. 7, pp. 523-32, 2008.
30. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, et al. (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 204:1257–1265.;
31. G. Borsellino, M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Hopner, D. Centonze, G. Bernardi, M. L. Dell'Acqua, P. M. Rossini, L. Battistini, O. Rotzschke, and K. Falk, "Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression," *Blood,* vol. 110, no. 4, pp. 1225-32, 2007.
32. A. Tanaka, and S. Sakaguchi, "Regulatory T cells in cancer immunotherapy," Cell Res, vol. 27, no. 1, pp. 109-118, 2017.
33. M. J. Smyth, G. P. Dunn, and R. D. Schreiber, "Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity," Adv Immunol, vol. 90, pp. 1-50, 2006.
34. C. Baecher-Allan, J. A. Brown, G. J. Freeman, and D. A. Hafler, "CD4+CD25high regulatory cells in human peripheral blood," *J Immunol,* vol. 167, no. 3, pp. 1245-53, 2001.

35. E. M. Shevach, "From vanilla to 28 flavors: multiple varieties of T regulatory cells," *Immunity,* vol. 25, no. 2, pp. 195-201, 2006

36. Safinia N, Scotta C, Vaikunthanathan T, Lechler RI, Lombardi G. Regulatory T Cells: Serious Contenders in the Promise for Immunological Tolerance in Transplantation. Front Immunol. 2015;6:438. Published 2015 Aug 31. doi:10.3389/fimmu.2015.00438

37. M. Suzuki, C. Konya, J. J. Goronzy, and C. M. Weyand, "Inhibitory CD8+ T cells in autoimmune disease," Hum Immunol, vol. 69, no. 11, pp. 781-9, 2008.

38. D. Fenoglio, F. Ferrera, M. Fravega, P. Balestra, F. Battaglia, M. Proietti, C. Andrei, D. Olive, L. C. Antonio, F. Indiveri, and G. Filaci, "Advancements on phenotypic and functional characterization of non-antigen-specific CD8+CD28- regulatory T cells," *Hum Immunol,* vol. 69, no. 11, pp. 745-50, 2008.

39. G. Filaci, D. Fenoglio, and F. Indiveri, "CD8(+) T regulatory/suppressor cells and their relationships with autoreactivity and autoimmunity," Autoimmunity, vol. 44, no. 1, pp. 51-7, 2011.

40. G. Filaci, M. Fravega, D. Fenoglio, M. Rizzi, S. Negrini, R. Viggiani, and F. Indiveri, "Non-antigen specific CD8+ T suppressor lymphocytes," Clin Exp Med, vol. 4, no. 2, pp. 86-92, 2004.

41. A. Parodi, F. Battaglia, F. Kalli, F. Ferrera, G. Conteduca, S. Tardito, S. Stringara, F. Ivaldi, S. Negrini, G. Borgonovo, A. Simonato, P. Traverso, G. Carmignani, D. Fenoglio, and G. Filaci, "CD39 is highly involved in mediating the suppression activity of tumor-infiltrating CD8+ T regulatory lymphocytes," Cancer Immunol Immunother, vol. 62, no. 5, pp. 851-62, 2013.

42. Grégoire V, Lefebvre JL, Licitra L, Felip E; EHNS-ESMO-ESTRO Guidelines Working Group. Squamous cell carcinoma of the head and neck: EHNS-ESMO-ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2010;21 Suppl 5:v184-v186. doi:10.1093/annonc/mdq185
43. Vermorken JB, Mesia R, Rivera F, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359(11):1116-1127. doi:10.1056/NEJMoa0802656
44. Magrini SM, Buglione M, Corvò R, et al. Cetuximab and Radiotherapy Versus Cisplatin and Radiotherapy for Locally Advanced Head and Neck Cancer: A Randomized Phase II Trial. J Clin Oncol. 2016;34(5):427-435. doi:10.1200/JCO.2015.63.1671;
45. von der Grün J, Rödel F, Brandts C, et al. Targeted Therapies and Immune-Checkpoint Inhibition in Head and Neck Squamous Cell Carcinoma: Where Do We Stand Today and Where to Go?. Cancers (Basel). 2019;11(4):472. Published 2019 Apr 3. doi:10.3390/cancers11040472
46. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354(6):567-578. doi:10.1056/NEJMoa053422
47. Vatner RE, Cooper BT, Vanpouille-Box C, Demaria S, Formenti SC. Combinations of immunotherapy and radiation in cancer therapy. Front Oncol. 2014;4:325. Published 2014 Nov 28. doi:10.3389/fonc.2014.00325
48. Wang SJ, Haffty B. Radiotherapy as a New Player in Immuno-Oncology. Cancers (Basel). 2018;10(12):515. Published 2018 Dec 14. doi:10.3390/cancers10120515

49. Goto T. Radiation as an In Situ Auto-Vaccination: Current Perspectives and Challenges. Vaccines (Basel). 2019;7(3):100. Published 2019 Aug 26. doi:10.3390/vaccines7030100

50. Loi M, Desideri I, Greto D, et al. Radiotherapy in the age of cancer immunology: Current concepts and future developments. Crit Rev Oncol Hematol. 2017;112:1-10. doi:10.1016/j.critrevonc.2017.02.002

51. Vacchelli E, Vitale I, Tartour E, et al. Trial Watch: Anticancer radioimmunotherapy. Oncoimmunology. 2013;2(9):e25595. doi:10.4161/onci.25595

52. Rodríguez-Ruiz ME, Vanpouille-Box C, Melero I, Formenti SC, Demaria S. Immunological Mechanisms Responsible for Radiation-Induced Abscopal Effect. Trends Immunol. 2018 Aug;39(8):644-655. doi: 10.1016/j.it.2018.06.001. Epub 2018 Jul 11. PMID: 30001871; PMCID: PMC6326574

53. Galluzzi L, Vitale I, Warren S, Adjemian S, Agostinis P, Martinez AB, Chan TA, Coukos G, Demaria S, Deutsch E, Draganov D, Edelson RL, Formenti SC, Fucikova J, Gabriele L, Gaipl US, Gameiro SR, Garg AD, Golden E, Han J, Harrington KJ, Hemminki A, Hodge JW, Hossain DMS, Illidge T, Karin M, Kaufman HL, Kepp O, Kroemer G, Lasarte JJ, Loi S, Lotze MT, Manic G, Merghoub T, Melcher AA, Mossman KL, Prosper F, Rekdal Ø, Rescigno M, Riganti C, Sistigu A, Smyth MJ, Spisek R, Stagg J, Strauss BE, Tang D, Tatsuno K, van Gool SW, Vandenabeele P, Yamazaki T, Zamarin D, Zitvogel L, Cesano A, Marincola FM. Consensus guidelines for the definition, detection and interpretation of immunogenic cell death. J Immunother Cancer. 2020 Mar;8(1):e000337. doi: 10.1136/jitc-2019-000337. Erratum in: J Immunother Cancer. 2020 May;8(1): PMID: 32209603; PMCID: PMC7064135.

54. Formenti SC, Demaria S. Combining radiotherapy and cancer immunotherapy: a paradigm shift. J Natl Cancer Inst. 2013 Feb 20;105(4):256-65. doi: 10.1093/jnci/djs629. Epub 2013 Jan 4. PMID: 23291374; PMCID: PMC3576324.

55. Fenoglio D, Ferrera F, Fravega M, Balestra P, Battaglia F, Proietti M, Andrei C, Olive D, Antonio LC, Indiveri F, Filaci G. Advancements on phenotypic and functional characterization of non-antigen-specific CD8+CD28- regulatory T cells. Hum Immunol. 2008 Nov;69(11):745-50. doi: 10.1016/j.humimm.2008.08.282. Epub 2008 Sep 29. PMID: 18832002.

56. de Goeje PL, Smit EF, Waasdorp C, Schram MTB, Kaijen-Lambers MEH, Bezemer K, de Mol M, Hartemink KJ, Nuyttens JJME, Maat APWM, Hegmans JPJJ, Hendriks RW, Senan S, Aerts JGJV. Stereotactic Ablative Radiotherapy Induces Peripheral T-Cell Activation in Patients with Early-Stage Lung Cancer. Am J Respir Crit Care Med. 2017 Nov 1;196(9):1224-1227. doi: 10.1164/rccm.201610-2178LE. PMID: 28345951.

57. Zhang T, Yu H, Ni C, Zhang T, Liu L, Lv Q, Zhang Z, Wang Z, Wu D, Wu P, Chen G, Wang L, Wei Q, Huang J, Wang X. Hypofractionated stereotactic radiation therapy activates the peripheral immune response in operable stage I non-small-cell lung cancer. Sci Rep. 2017 Jul 7;7(1):4866. doi: 10.1038/s41598-017-04978-x. PMID: 28687760; PMCID: PMC5501824.

58. Wang XB, Wu DJ, Chen WP, Liu J, Ju YJ. Impact of radiotherapy on immunological parameters, levels of inflammatory factors, and clinical prognosis in patients with esophageal cancer. J Radiat Res. 2019 May 1;60(3):353-363. doi: 10.1093/jrr/rrz006. PMID: 31034571; PMCID: PMC6530619.

59. van Meir H, Nout RA, Welters MJ, Loof NM, de Kam ML, van Ham JJ, Samuels S, Kenter GG, Cohen AF, Melief CJ, Burggraaf J, van Poelgeest MI, van der Burg SH. Impact of (chemo)radiotherapy on immune cell composition and function in cervical cancer patients. Oncoimmunology. 2016 Dec 23;6(2):e1267095. doi: 10.1080/2162402X.2016.1267095. PMID: 28344877; PMCID: PMC5353924.

60. Balázs K, Kis E, Badie C, Bogdándi EN, Candéias S, Garcia LC, Dominczyk I, Frey B, Gaipl U, Jurányi Z, Kocsis ZS, Rutten EA, Sáfrány G, Widlak P, Lumniczky K. Radiotherapy-Induced Changes in the Systemic Immune and Inflammation Parameters of Head and Neck Cancer Patients. Cancers (Basel). 2019 Sep 6;11(9):1324. doi: 10.3390/cancers11091324. PMID: 31500214; PMCID: PMC6770727.